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(54) ELECTROCHEMICAL BIOSENSORS AND PROCESS FOR THEIR PREPARATION

ELEKTROCHEMISCHE BIOSENSOREN UND VERFAHREN ZU IHRER HERSTELLUNG

BIOCAPTEURS ELECTROCHIMIQUES ET LEUR PROCEDE DE PRODUCTION

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Description

[0001] The present invention concerns new electrochemical biosensors based on composite transducers containing solid binding makers, prepared by incorporating a biocatalyst into the bulk of said transducers or by applying a biocatalytic layer onto the surface of said transducers. The biosensors of the invention are useful in the determination of the concentration of specific analytes in sample solutions or suspensions.

PRIOR ART DISCLOSURE

[0002] A biosensor is a device incorporating a biological sensing element either intimately connected to or integrated within a transducer. Its usual aim is to produce electronic signals which are proportional to the concentration of the specific substance which has to be determined.

[0003] Analytical advantages of biosensors consist in their specificity, sensitivity, simple manipulation, rapid response and consequent low costs of analysis. These specific and sensitive devices have been used in medical diagnostics, in monitoring food quality and freshness, in environmental monitoring, in fermentation and analytical control, and so on.

[0004] Electrochemical biosensors, especially amperometric ones, play a significant role in the applications of these devices. Amperometric biosensors of the second generation, based on redox reactions, are characterized by the use of chemical mediators instead of molecular oxygen. During the transformation of a substrate by a bioactive material, chemical mediators shuttle electrons from the redox centre of the biocatalyst itself to the indicating electrode and the corresponding amperometric signal is measured.

[0005] One of the most promising groups of chemical mediators consists of metallocenes, and in particular ferrocenes. The first successful enzyme electrode based on ferrocene was prepared by Cass et al. (*Anal. Chem.* 56, 667-671, 1984); in this glucose sensitive sensor, the electrode was prepared by soaking a spongy carbon foil with 1,1'-dimethylferrocene and the enzyme glucose oxidase was chemically immobilized on the surface of this electrode.

[0006] Later on, Dicks et al. (*Ann. Biol. Clin.* 47, 607-619, 1989) prepared a gold microelectrode covered with a polypyrrol film, on which glucose oxidase and ferrocene were adsorbed. However, these electrodes, having chemical mediators adsorbed on the surface of the device, show poor stability due to the leaking of the mediators out of the transducer.

[0007] Since the late 1980s, intensive research activities were devoted to the development of biosensors based on Carbon Paste Electrodes (CPE); a carbon paste electrode is a mixture of electrically conducting graphite or carbon with a pasting liquid, e.g. paraffin oil, silicon oil, Nujol etc.

[0008] This kind of electrodes has the advantage of allowing bulk modification of the electrode material with biocatalysts, as well as with other advantageous components, essential for the effective functioning of the device. Furthermore, bulk modification allows to create sensors with renewable surfaces, so that each measurement can be performed on a new surface of the electrode, thus avoiding the drawbacks due to previous measurement residuals.

[0009] A CPE is commonly prepared by mixing carbon or graphite powder with a biocatalyst, a chemical mediator and optionally a co-factor, and by finally adding one of the above mentioned pasting liquids. The thus obtained paste is packed in a suitable tube, so to obtain a disc electrode (L. Gorton, *Electroanalysis*, 7, 23-45, 1995). These biosensors can be used for the detection of different analytes, such as glucose, fructose, galactose, ethanol, glycerol, aminoacids, lactate, xanthines etc., and are based on the corresponding oxidases and co-factor dependent dehydrogenases.

[0010] In EP-A-400918 enzyme sensors are produced by mixing liquid binders like liquid paraffin with graphite or carbon.

[0011] J. Electroanal. Chem., 1984, 176, 169-182 describes the influence of different pasting liquids on the capacity of carbon paste electrodes.

[0012] US-A-5269891 describes electrode devices containing non polar pasting materials. WO-A-9424548 refers to a flow injection analysis biosensor based on graphite paste modified with tetracyanoquinodimethane.

[0013] Nevertheless, CPE based biosensors show the following practical drawbacks and limitations:

- i) poor mechanical properties, due to their paste creamy character imparted by the pasting liquid, often leading to easy disintegration of the system;
- ii) poor compatibility of the paste with the biocatalytic enzyme, due to their opposite chemical properties (an enzyme is usually hydrophilic, while paste is strongly hydrophobic), often leading to phase separation (biocatalyst/transducer);
- iii) leaking of the mediator out of the CPE, due to poor compatibility of CPE with the mediator.

[0014] Finally, the above mentioned features of CPE hinder or significantly reduce the electron transfer from the biocatalytic site to the electrode.

[0015] In alternative to the use of paste matrices for the fabrication of composite enzyme electrodes, direct modification of enzymes with chemical mediators was carried out by A. E. G. Cass and M. H. Smit (*Trends in Electrochemical Biosensors*, G. Costa e S. Miertus Ed., *World Sci. Publ.*, 25-42, 1992); in order to prevent the mediator leaking out of the transducer, peroxidase was covalently modified with ferrocenyl groups, by derivatizing the enzyme chains near the active redox centre.

[0016] Furthermore, Karube et al. recently described

enzyme-immobilized electrodes based on electrically conducting enzymes (EPA 0 563 795), i.e. enzymes to which electrical conductivity is imparted by covalently linking a chemical mediator. The electrical conductivity of such enzymes varies depending upon the amount of presence of the substrate to be detected in a specimen sample. Chemical mediators can be attached through a covalent bond to enzymes, such as oxidases or dehydrogenases, either to the proteic body (for example, to an aminic group of a lysine residue) or to a side chain of the enzyme (for example, to an oligosaccharide chain, to an alkyl chain, to a peptidic chain linked to the main chain, etc.).

[0017] Nevertheless, the covalent attachment of a mediator to the enzyme has the great disadvantage of changing the electrochemical properties of the mediator itself, consequently reducing its mobility and affecting its reaction rate with the enzyme.

[0018] Moreover, the procedures and operative conditions used in the modification of enzymes are rather drastic for biocatalysts; chemical modification can significantly reduce enzyme activity and stability, leading to a consequent decrease or total loss of activity, when naturally low-stable enzymes are used. Furthermore, said chemical modification leads to an increase of production costs.

[0019] Therefore, it is felt the need of devising alternative electrochemical biosensors, endowed with higher stability and efficacy with respect to the known devices.

SUMMARY OF THE INVENTION

[0020] Now, the Applicant has unexpectedly found new electrochemical biosensors based on composite transducers, comprising at least a solid binding maker, as defined hereunder, able to overcome the drawbacks of the prior art.

[0021] The biosensors of the invention comprise:

- at least an electro-conducting material, in the form of powder or grains;
- at least a chemical mediator;
- optionally, a substance capable of sorption of said chemical mediator;
- a solid binding maker, which is a compound in solid state at room temperature, selected from the group of chemical compounds consisting of: linear or branched, saturated or unsaturated hydrocarbons, containing from 12 to 60 carbon atoms, preferably from 12 to 30 carbon atoms, optionally substituted with at least a group selected from -OH, -SH, -NH₂, -CO-, -CHO, -SO₃H, -COOH, -OR₁, -SR₁, -NR₁R₂ and -COOR₁, wherein R₁ and R₂ are independently hydrocarbon groups C₁-C₃₀, optionally containing one or more heteroatoms; esters of fatty acids with glycerol; and esters of fatty acids with cholesterol; wherein said solid binding maker

which is in solid state at room temperature, having a melting point comprised between 25°C and 200°C, and

e) at least a biocatalyst, selected from the group consisting of enzymes, cells, cellular components, tissues, immunoproteins and DNA.

[0022] In the biosensors of the invention, said biocatalyst (e) can be either incorporated into the body of the composite transducer, made up of components (a)-(d), or applied onto the surface of said composite transducer, in the form of a film layer.

[0023] The biosensor of the invention can be optionally covered by a suitable membrane. When said biocatalyst is an enzyme requiring the presence of the corresponding co-factor, the biosensors of the invention comprise even said co-factor.

[0024] Furthermore, the present invention concerns a process for the preparation of a biosensor as described above, comprising the following steps:

- mixing said electro-conducting material with said chemical mediator;
- optionally mixing the mixture obtained in step (1) with said substance capable of sorption of the chemical mediator;
- optionally mixing the mixture obtained in step (1) or (2) with said biocatalyst;
- suitably mixing the mixture obtained in step (1), (2) or (3) with said solid binding maker;
- introducing and optionally pressing the mixture obtained in step (4) in a suitable holder, thus obtaining a compact form;
- when step (3) is not worked out, applying a biocatalytic layer onto the surface of the transducer obtained in step (5);
- optionally covering the biosensor obtained in step (5) or (6) with a suitable membrane.

[0025] A further object of the present invention is a procedure for the determination of analytes concentration in sample solutions or suspensions, comprising applying suitable electrode potential, contacting the biosensor of the invention with said sample solutions or suspensions and finally measuring current changes, which are proportional to the concentration of the analyte.

DESCRIPTION OF THE DRAWINGS

[0026] Figure 1 shows a preferred embodiment of a biosensor according to the present invention, wherein the composite transducer 2 is cylindrically shaped and is allocated in an envelope 1, made up of a glass or plastic cylinder; a membrane or net 4 can fix the biocatalyst to the transducer 2. In Figure 1A, said biocatalyst is applied onto the surface of said transducer 2 in the form of a layer 3; in Figure 1B, said biocatalyst 5 is incorpo-

rated into the bulk of the transducer 2.

[0027] Figures 2, 3, and 13 are cyclic voltammograms obtained with transducers prepared respectively in examples 1, 2 and 12.

[0028] Figure 4 shows the relationship between current value and glucose concentration with the "bulk" biosensor prepared in example 3.

[0029] Figure 5 shows the relationships between polarization time and the relative sensitivity of the biosensor of example 3 (curve 1) and of a similar biosensor, not comprising a solid binding maker, prepared as described in the comparative example 4 (curve 2).

[0030] Figures 6-8, 10, 14 and 19 show the relationship between current values and glucose concentrations with the biosensors prepared respectively in examples 5-7, 9, 18 and 19.

[0031] Figures 9, 11, 12 and 16-18 show the relationship between current values and ethanol concentrations with the biosensors prepared respectively in examples 8, 10, 11 and 15-17.

[0032] Figure 15 shows the relationship between current value and fructose concentration with the biosensor prepared in example 14.

DETAILED DESCRIPTION OF THE INVENTION

[0033] The characteristics and the advantages of the new electrochemical biosensors based on new composite transducers, according to the present invention, will be better reported in the following detailed description.

[0034] The biosensors according to the present invention are capable of quantitatively determining a specific analyte contained in a sample, such as a solution or a suspension, in a rapid and easy manner; furthermore, said biosensors show good mechanical properties, particularly compactness and plasticity, and do not disintegrate during the use.

[0035] These advantages are mainly due to the use of solid binding maker as essential innovative component of the transducer, giving solid compact mixtures with high stability, efficient communication between redox center and electrode, stability of the biocatalyst and retention of the chemical mediator, thus overcoming carbon paste drawbacks of the prior art.

[0036] According to a preferred embodiment of the biosensors according to the present invention, said electro-conducting material, said chemical mediator, said solid binding maker and optionally said substance capable of sorption of said chemical mediator are mixed together and pressed to give the composite transducer, in the form of a compact solid mixture. The biocatalyst can be either incorporated into the bulk of said composite transducer or applied onto the transducer surface in the form of a film layer.

[0037] According to another embodiment of the biosensors of the invention, the composite transducer does not contain the chemical mediator, which is directly added to the sample solution or suspension to be tested.

[0038] Alternatively, said chemical mediator can be contained, at the same time, in the composite transducer and in the sample solution or suspension.

[0039] Said electro-conducting material, in the form of powder or grains, is preferably selected from the group consisting of metals, such as gold, platinum, palladium, iridium, and their alloys, carbon and graphite. This electro-conducting material has preferably a granulometry ranging from 0.05 to 200 µm.

[0040] Said chemical mediator is one of the natural and synthetic mediators commonly used in the biosensors known in art, and is preferably selected from the group consisting of cytochromes, quinones, aminophenols, electronacceptor aromatic compounds (e.g. TTF=tetrathiafulvalene and NMP=N-methylphenazidium), electondonor aromatic compounds (e.g. TCNQ=tetracyano-p-quinodimethane), organic conducting salts (e.g. TTF.TCNQ = tetrathiafulvalene 7,7,8,8-tetracyano-p-quinodimethane and NMP.TCNQ = N-methylphenazinium 7,7,8,8-tetracyano-p-quinodimethane), organic dyes, metallocenes, organometallic complexes of Os, Ru and V, inorganic complexes of Fe. More preferably, said chemical mediator is ferrocene, 1,1'-dimethyl-ferrocene, hexacyanoferrate (II) or hexacyanoferrate (III).

[0041] Said substance capable of sorption of the chemical mediator is any substance having adsorption and ionic-exchange properties, preferably selected from the group consisting of silica, alumina, zeolites and Nafion®. Said substance is optionally contained in the composite transducer of the biosensor according to the present invention; its presence is preferred in case of prolonged and multiple use of the biosensor itself.

[0042] Said solid binding maker is a compound which is in solid state at room temperature, having a melting point comprised between 25 and 200°C, and more preferably between 35 and 90°C (in particular, having a melting point of 25-90°C for bulk biosensors, and of 25-200°C for biosensors having a biocatalytic layer applied onto their surface).

[0043] Said solid binding maker belongs to one of the following classes:

- i) linear or branched, saturated or unsaturated hydrocarbons, containing from 12 to 60 carbon atoms, preferably from 12 to 30 carbon atoms, optionally substituted with at least a polar group selected from -OH, -SH, -NH₂, -CO-, -CHO, -SO₃H, -COOH, -OR₁, -SR₁, -NR₁R₂ and -COOR₁, wherein R₁ and R₂ are independently hydrocarbon groups C₁-C₃₀, optionally containing one or more heteroatoms;
- ii) esters of fatty acids with glycerol;
- iii) esters of fatty acids with cholesterol.

[0044] When the solid binding maker belongs to class (i), it is preferably hexadecanol, hexadecanone, tetradecylamine, eicosane or tetracosane.

[0045] When the solid binding maker belongs to class

(ii), it is preferably a mono, bi or triester of glycerol with fatty acids containing from 12 to 24 carbon atoms; more preferably, said solid binding maker is monostearoyl glycerol or lecithin.

[0046] When the solid binding maker belongs to class (iii), it is preferably an ester of cholesterol with a fatty acid containing from 12 to 24 carbon atoms; more preferably, said solid binding maker is cholesteryl myristate, cholesteryl stearate or cholesteryl oleate.

[0047] Said solid binding maker plays a leading role in the composition of the composite transducer of the biosensors of the invention; in fact, said compound is capable of imparting improved mechanical properties to the transducer itself, especially compactness and plasticity, in comparison with the common carbon pastes of the prior art, where pasting liquids were used. Furthermore, said solid binding makers ensure a higher compatibility with the biocatalyst and the chemical mediator; moreover, they are capable of exerting an action in support of the substances capable of sorption of the chemical mediator (the presence of which is only optional), thus accounting to physical immobilisation of both the biocatalyst and the chemical mediator.

[0048] Said solid binding maker is also able to secure a suitable molecular environment within the transducer body, which allows an efficient electron transfer among the redox centre of the biocatalyst, the chemical mediator and the conducting material, and subsequently great effectivity of the biocatalytic process, high conductivity and stability, during both storage and use.

[0049] Finally, thanks to said solid binding makers, it is possible to obtain solid-state transducers according to the desired forms and shapes; the absence of these binding makers leads to a drastic reduction of the biosensor performance and to the loss of its mechanical properties.

[0050] Said biocatalyst can be constituted of one or more enzymes able to catalyse a redox reaction, cells, cellular components, tissues, immunoproteins or DNA.

[0051] Preferably, said biocatalyst belongs to one of the following classes:

- enzymes preferably selected from the group consisting of glucose oxidase, galactose oxidase, glycolate oxidase, alcohol oxidase, cholesterol oxidase, polyphenol oxidase, ascorbate oxidase, lipoxygenase, lipoxidase, peroxidase, catalase, xanthine oxidase, pyruvate oxidase, β -galactosidase, invertase, cholinesterase, citrate lyase, amylases and mixtures thereof;
- enzymes which require the presence of a co-factor, in association with said co-factor, preferably selected from the group consisting of glucose dehydrogenase, alcohol dehydrogenase, fructose dehydrogenase, malate dehydrogenase, lactate dehydrogenase, mannitol dehydrogenase, glycerol dehydrogenase, isocitrate dehydrogenase, galactose dehydrogenase, glucose phosphate dehydrogenase,

tartrate dehydrogenase and mixtures thereof; - cells, preferably selected from the group consisting of *Gluconobacter oxidans*, *Escherichia coli*, *Aspergillus niger*, *Pseudomonas fluorescens*, *Trichosporon brassicae*, *Saccharomyces cerevisiae*, *Brevicacterium lactofermentum*, *Enterobacter agglomerans*, *Leuconostoc mesenteroides*, *Nocardia erythropolis* and mixtures thereof;

- immunoproteins and immunosystems based on antibodies labelled by enzymes. When the enzyme requires the presence of a co-factor, said co-factor is preferably selected from the group consisting of NAD, NADH, NADP, NADPH, FAD, FMM and quinones. The co-factor is contained, as well as the corresponding enzyme, in the biosensor of the invention.

[0052] In particular, according to a specific embodiment, both the enzyme and the corresponding co-factor can be contained in the bulk of said biosensor; alternatively, both the enzyme and the corresponding co-factor can be applied onto the surface of said transducer, in the form of a layer; otherwise, the enzyme can be applied as a layer onto the surface of said transducer, while the corresponding co-factor is incorporated in the body of the transducer itself.

[0053] The biosensors of the invention can be optionally covered with a suitable membrane. Said membrane can be one of the different types of dialysis membranes known in the state of the art, preferably based on cellulose acetate or cellophane®, or it can be a membrane based on nitrocellulose, PVC, Teflon®, Nylon, polycarbonates or polyesters. The quantitative composition of the above described composite transducer, with reference to 100 parts by weight of the composite transducer itself, composed of components (a)-(d), is as follows:

- a) electro-conducting material: from 20 to 80%, and preferably from 30 to 60% by weight;
- b) chemical mediator: from 0.5 to 30%, and preferably from 1 to 10% by weight;
- c) substance capable of sorption of said chemical mediator: from 0 to 30%, and preferably from 1 to 20% by weight. When said substance is silica, alumina or zeolites, from 0 to 30%, and preferably from 5 to 20% by weight; when said substance is Nafion®, from 0 to 5%, and preferably from 1 to 3% by weight;
- d) solid binding maker: from 10 to 80%, and preferably from 30 to 60% by weight. In the "bulk" biosensors according to the present invention, the biocatalyst content ranges from 1 to 30% by weight, with respect to the weight of the composite transducer. When said biocatalyst is an enzyme requiring the presence of a co-factor, the quantity of co-factor ranges from 0.5 to 30%, and preferably from 3 to 10% by weight of the composite transducer.

[0054] The biosensor of the present invention can be prepared according to different shapes and geometries. Figure 1 shows a cylindrical biosensor; however, it can be prepared even in other geometrical shapes, such as in the form of parallelepipedon, sphere, plate, film, screen printed layer and so on.

[0055] The composite transducer can be prepared according to the following steps:

- 1) said electro-conducting material is mixed with said chemical mediator;
- 2) the thus obtained mixture is optionally mixed with said substance capable of sorption of the chemical mediator;
- 3) the mixture obtained in step (1) or (2) is suitably mixed with said solid binding maker;
- 4) the mixture obtained in stage (3) is introduced and optionally pressed in a compact form, thus obtaining the composite transducer. Said composite transducer can be smoothed at the base, for example on a sheet of common paper.

[0056] In step (3), the admixing of the solid binding maker has to be carried out by preferably mixing according to one of the following procedures:

- vigorous mechanical mixing of all solid components;
- mixing in the presence of a suitable solvent which is then evaporated, preferably chloroform or ethanol;
- mixing the mixture obtained in step (1) or (2) with the solid binding maker in melted state.

[0057] For the preparation of a preferred embodiment of the biosensor of the invention, a biocatalytic layer can be applied onto the base surface of said composite transducer, as shown in Figure 1A.

[0058] Alternatively, according to another embodiment of the biosensor of the invention, the biocatalyst can be directly incorporated in the body of the composite transducer, as shown in Figure 1B, by its admixing in step (3), thus obtaining a "bulk" biosensor.

[0059] Finally, the biosensors of the invention can be covered by a suitable membrane, as described above.

[0060] The biosensors according to the present invention show high specificity and sensitivity, and can be advantageously used in human and veterinary diagnostics, in industrial processes, in the quality control of food, in biotechnology, in the pharmaceutical industry, in the environmental monitoring and so on.

[0061] The procedure for the detection and quantification of analytes, in sample solutions or suspensions, comprises applying suitable electrode potential, contacting a biosensor according to the present invention, as described above, with said analytes solutions or suspensions and finally measuring current changes, which are proportional to the concentration of the analyte.

[0062] The following examples are reported for illustrative, but not limitative purposes.

Example 1

Preparation of a transducer containing monostearoyl glycerol as solid binding maker.

[0063] 1 g of synthetic graphite powder (Aldrich, Cat. No. 28, 286-3, 1994) was added to a solution containing 62 mg of 1,1'-dimethyl-ferrocene (Aldrich) in 5 ml of chloroform. The mixture was stirred at room temperature until chloroform was completely evaporated. 180 mg of the above mixture were mixed thoroughly with alumina (20 mg, 1 µm average particle size). Monostearoyl glycerol (200 mg) was added and mixed.

[0064] The thus obtained mixture was introduced in a bottomless glass tube, having an inner diameter of 2 mm, and pressed with a glass rod. The surface of the electrode was smoothed on a sheet of ordinary paper. The electric contact was made through a copper wire. The obtained transducer was tested by cyclic voltammetry under the following conditions: 0.1 M sodium phosphate, pH = 6.5, saturated with nitrogen, scan rate 100mV/s, instrumentation Amel - model 433V, saturated calomelan reference electrode (SCE), Pt counter electrode. The cyclic voltammogram is shown in Figure 2.

Example 2

Preparation of a transducer containing monostearoyl glycerol as solid binding maker.

[0065] Graphite powder was added to 1,1'-dimethyl-ferrocene, as described in Example 1. The obtained product (200 mg) was thoroughly mixed with Nafion® (100 µl of 5%, Aldrich, Cat. No. 27,470-4, 1994) and then with monostearoyl glycerol (200 mg). The obtained mixture was introduced in a bottomless glass tube and tested under the conditions described in Example 1. The cyclic voltammogram is shown in Figure 3.

Example 3

Preparation of a "bulk" biosensor for the determination of glucose, containing monostearoyl glycerol as solid binding maker.

[0066] Graphite powder was added to 1,1'-dimethyl-ferrocene, as described in Example 1. The obtained mixture (200 mg) was thoroughly mixed with Nafion® (100 µl of 5%, Aldrich, Cat. No. 27,470-4, 1994). The obtained product (190 mg) was thoroughly mixed with glucose oxidase (10 mg, Sigma, Cat. No. G -7016) and then with monostearoyl glycerol (200 mg). The mixture was introduced in a bottomless glass tube, having an inner diameter of 2 mm, pressed with a metal rod and the base surface was smoothed on a sheet of ordinary

paper. The thus obtained biosensor was covered with a dialysis membrane (Spectra/Por® MWCO 6,000-8,000) by means of an O-ring. The biosensor was polarized at 250 mV vs. SCE. The response of the biosensor was recorded in a buffer solution (0.1 M sodium phosphate, pH = 6.5), under nitrogen stream (to avoid the effect of oxygen), for several glucose concentrations. The relationship between glucose concentration and current change is shown in Figure 4. The response was linear up to the concentration of 12 mM. The equation for the linear part of the response is $I = 0.013 + 0.364c$, where I is the current value [μ A] and c is the glucose concentration in mM, the coefficient of regression r being 0,9997.

[0067] The obtained biosensor underwent also stability tests. It was polarized at potential of 250 mV vs. SCE in a buffered solution of glucose (4 mM), saturated with nitrogen, and the response was recorded after various polarization times. The relationship between relative sensitivity and polarization time is shown in Figure 5 (curve 1).

Comparative example 4

Preparation of the "bulk" biosensor of example 3, In the absence of monostearoyl glycerol as solid binding maker.

[0068] A biosensor was prepared as described in Example 3, without adding monostearoyl glycerol as solid binding maker. In order to confer consistency to the transducer, ordinary wax was added to the mixture instead of monostearoyl glycerol. The sensor underwent stability tests, as reported in Example 3. Figure 5 (curve 2) shows that, in the absence of a suitable solid binding maker, the stability of the biosensor is significantly reduced.

Example 5

Preparation of a "bulk" biosensor for the determination of glucose, containing cholesteryl oleate as solid binding maker.

[0069] Graphite powder was added to 1,1'-dimethyl-ferrocene, as described in Example 1. The obtained product (45 mg) was thoroughly mixed with alumina (10 mg), glucose oxidase (4 mg) and cholesteryl oleate (42 mg, Sigma, Cat. No. C-9253, 1994). The obtained mixture was introduced in a bottomless glass tube, having an inner diameter of 2 mm, and pressed with a metal bar. The base surface was then smoothed on a sheet of ordinary paper. The thus obtained biosensor was covered with a dialysis membrane (Spectra/Por®, MWCO 6,000-8,000) by means of an O-ring. The electrode was polarized at 300 mV vs. SCE. The response of the electrode was recorded in a buffer solution (0.1 M sodium phosphate, pH = 6.5), under nitrogen stream (to avoid

the effect of oxygen), at several glucose concentrations. The relationship between glucose concentration and current change is shown in Figure 6.

5 Example 6

Preparation of a "bulk" biosensor for the determination of glucose, containing lecithin as solid binding maker.

[0070] Graphite powder was added to 1,1'-dimethyl-ferrocene, as described in Example 1.
 [0071] The obtained product (45 mg) was thoroughly mixed with alumina (10 mg), glucose oxidase (4 mg) and lecithin (30 mg, Fluka, Cat. No. 61755, 1993-1994). The obtained mixture was introduced in a glass tube, pressed and smoothed, and the obtained biosensor was covered with a dialysis membrane, as described above. The obtained electrode was polarized at 300 mV vs. SCE. The response of the biosensor was recorded in a buffer solution (0.1 M sodium phosphate, pH = 6.5), under nitrogen stream (to avoid the effect of oxygen), at several glucose concentrations. The relationship between glucose concentration and current change is shown in Figure 7.

Example 7

Preparation of a "bulk" biosensor for the determination of glucose, containing monostearoyl glycerol as solid binding maker.

[0072] 1 g of graphite powder was added to a solution of 62 mg of tetrathiafulvalene (Aldrich, Cat. No. 18,318-0, 1995) in 5 ml of chloroform, as described in example 1. 45 mg of the obtained product were thoroughly mixed with alumina (10 mg), glucose oxidase (4 mg), and monostearoyl glycerol (45 mg). The obtained mixture was introduced in a glass tube, pressed and smoothed, and the obtained biosensor was covered with a dialysis membrane, as described above. The obtained electrode was polarized at 300 mV vs. SCE. The response of the electrode was recorded in a buffer solution (0.1 M sodium phosphate, pH = 6.5), under nitrogen stream (to avoid the oxygen influence), at several glucose concentrations. The relationship between glucose concentration and current change is shown in Figure 8.

50 Example 8

Preparation of a "bulk" biosensor for the determination of ethanol, containing monostearoyl glycerol as solid binding maker.

1. Preparation of *Gluconobacter oxydans* cells

[0073] Cells of *Gluconobacter oxydans* CCM 1783

were grown in a culture medium (100 g of glycerol, 5 g of yeast extract per 1 l, tap water), at 30°C. In the late exponential phase ($OD_{650} = 0.6$), the suspension was centrifuged (5 min at 4,500 rpm), washed three times with a solution of NaCl (10 g/l) and dried under reduced pressure.

2. Preparation of biosensor

[0074] Graphite powder was added to 1,1'-dimethyl-ferrocene, as described in Example 1.

[0075] The obtained product (200 mg) was thoroughly mixed with Nafion® (100 µl of 5%, Aldrich, Cat. No. 27,470-4, 1994). The mixture (120 mg) was thoroughly mixed with 9 mg of the dried biomass of *Gluconobacter oxydans* and then with monostearoyl glycerol (120 mg).

[0076] The obtained mixture was introduced in a glass tube, pressed and smoothed, and the obtained biosensor was covered with a dialysis membrane, as described above. The biosensor was polarized at 250 mV vs. SCE. The response of the biosensor was recorded in a buffer solution (0.1 M sodium phosphate, pH=6.5), under nitrogen stream (to avoid the effect of oxygen), at several ethanol concentrations. The relationship between ethanol concentration and current change is shown in Figure 9.

Example 9

Preparation of a biosensor for the determination of glucose, based on a layer of glucose oxidase and containing monostearoyl glycerol as solid binding maker.

[0077] A transducer was prepared as described in Example 2. Glucose oxidase (2 µl, 100 mg/ml) was then applied on the base surface of the transducer. After drying, the obtained biosensor was covered with a dialysis membrane (Spectra/Por® MWCO 6,000-8,000), by means of an O-ring. The obtained biosensor was polarized at 250 mV vs. SCE. The response was recorded in a buffer solution (0.1 M sodium phosphate, pH = 6.5), under nitrogen stream (to avoid the effect of oxygen), at several glucose concentrations. The relationship between glucose concentration and current change is shown in Figure 10.

Example 10

Preparation of a biosensor for the determination of ethanol, based on a layer of cells of *Gluconobacter oxydans* and containing monostearoyl glycerol as solid binding maker.

1. Preparation of *Gluconobacter oxydans* cells

[0078] Cells of *Gluconobacter oxydans* CCM 1783 were grown under the conditions described in Example

5
8. In the late exponential phase ($OD_{650} = 0.6$), a part of the suspension (25 ml) was centrifuged (5 min at 4,500 rpm), washed three times with a solution of NaCl (10 g/l) and finally resuspended in the same NaCl solution (1 ml).

2. Preparation of biosensor

[0079] A disc of filter paper was put onto a Petri dish and the paper was soaked with NaCl solution. A disc of membrane for microfiltration (Millipore, HA 45 µm porosity, 5 mm diameter) was put onto said soaked filter paper and the suspension of *Gluconobacter oxydans* (10 µl) was applied onto the membrane. After suction of the liquid, the disc was put onto the base surface of the transducer, prepared as reported in Example 2, and fixed by means of a nylon net and of an O-ring. The biosensor was polarized at 250 mV vs. SCE. The response of the biosensor was recorded in a buffer solution (0.1 M sodium phosphate, pH = 6.5), under nitrogen stream (to avoid the effect of oxygen), at several ethanol concentrations. The relationship between ethanol concentration and current change is shown in Figure 11.

Example 11

Preparation of a "bulk" biosensor for the determination of ethanol, containing monostearoyl glycerol as solid bindig maker.

[0080] Graphite powder was added to 1,1'-dimethyl-ferrocene, as described in Example 1. The obtained mixture (40 mg) was thoroughly mixed with alumina (10 mg), NAD (5mg, Fluka, Cat. N° 43407, 1995-1996) and alcohol dehydrogenase (3 mg, Sigma Cat. N°A-7011, 1995). The thus prepared product was mixed with monostearoyl glycerol (42 mg). The mixture was introduced in a glass tube, pressed and smoothed, and the obtained biosensor was covered with a dialysis membrane, as described above. The obtained biosensor was polarized at 250 mV vs. SCE. The response of the biosensor was recorded in a buffer solution (0.5 M TRIS-HCl buffer, pH = 8.8), at several ethanol concentrations. The relationship between ethanol concentration and current change is shown in Figure 12.

Example 12

Preparation of a transducer containing cholesteryl myristate as solid binding maker.

[0081] 1 g of graphite powder was added to 62 mg of ferrocene, as described in Example 1.

[0082] Cholesteryl myristate (200 mg, Sigma, Cat.No. C-5076, 1994) was melted in a porcelain dish, immersed in an oil bath heated to 85 °C. The above modified graphite (150 mg) was added into the melted mass and thoroughly mixed. APVC tip (inner diameter 2 mm, outer

diameter 5 mm, length 20 mm) was used as a holder of electrode. Into said tip, a brass stick (diameter 2 mm, length 70 mm) was inserted to create cylindrical space (thickness 2-3 mm). The space was filled with the melted mass. The electrode was left to cool to room temperature and the excess of the material was cut out on a sand paper (type P 1000). The surface was then smoothed on a sheet of common paper. The transducer was tested by cyclic voltammetry, under the conditions described in Example 1. The voltammogram is shown in Figure 13.

Example 13

Preparation of a biosensor for the determination of glucose, based on a layer of glucose oxidase and containing cholesteryl myristate as solid bindig maker.

[0083] A solution of glucose oxidase (2 µl, 10 mg/ml) was put and spread over the surface of a transducer, prepared according to Example 12. After drying, the electrode was covered by a dialysis membrane (*Spectra/Por®*, MWCO 6,000-8,000), by means of an O-ring. The electrode was polarized at 300 mV vs. SCE. The response of the electrode was recorded in a buffer solution (0.2 M sodium phosphate buffer, pH = 8), for various glucose concentrations. The relationship between glucose concentration and current change is shown in Figure 14.

Example 14

Preparation of a biosensor for the determination of fructose, based on a layer of fructose dehydrogenase and containing cholesteryl myristate as solid bindig maker.

[0084] A solution of fructose dehydrogenase (1 µl, 18 mg/ml, Sigma, Cat.No. F-4892, 1995) was put and spread over the surface of a transducer, prepared according to Example 12. After drying, the electrode was covered by a dialysis membrane, as described above. The electrode was polarized at 300 mV vs. SCE. The response of the electrode was recorded in a buffer solution (0.2 M sodium phosphate buffer, pH = 5.8), for various fructose concentrations. The relationship between fructose concentration and current change is shown in Figure 15.

Example 15

Preparation of a biosensor for the determination of ethanol, containing cholesteryl myristate as solid bindig maker, supplementing the tested solutions with a chemical mediator.

[0085] NAD (8 mg) was dissolved in distilled water (1 ml) and graphite powder (74 mg) was added. The ob-

tained suspension was stirred for 30 minutes at room temperature. Water was then evaporated, under reduced pressure. The obtained product was added into melted cholesteryl myristate (120 mg), thoroughly mixed and then introduced into a PVC tip, under the conditions indicated in Example 12. The obtained electrode was left to cool to room temperature and the excess of the material was cut out on a sand paper (type P 1000). The surface was then smoothed on a sheet of common paper. A solutions of ADH (1 µl, 5 mg/ml) and diaphorase (1.5 µl, 50 mg/ml, Sigma, Cat.No. D-5540, 1995) were mixed and spread over the surface of the obtained transducer. After drying, the electrode was covered by a dialysis membrane, as described above. The obtained electrode was polarized at 300 mV vs. SCE. The response of the electrode was recorded in a buffer solution (0.5 M TRIS-HCl buffer, pH = 8.8), supplemented with 2 mM hexacyanoferrate (III), for various ethanol concentrations. The relationship between ethanol concentration and current change is reported in Figure 16.

Example 16

Preparation of a biosensor for the determination of ethanol, containing cholesteryl myristate as solid bindig maker, supplementing the tested solutions with a chemical mediator.

[0086] NAD (8 mg), diaphorase (6 mg) and ADH (2 mg) were dissolved in distilled water (1 ml) and graphite powder (70 mg) was added. The suspension was stirred for 30 minutes at room temperature. Water was then evaporated, under reduced pressure. The obtained product was added into melted cholesteryl myristate (125 mg), thoroughly mixed and then introduced into a PVC tip, under the conditions reported in Example 12. The obtained electrode was left to cool to room temperature and the excess of the material was cut out on a sand paper (type P 1000). The surface was then smoothed on a sheet of common paper and covered with a dialysis membrane, as described above. The electrode was polarized at 300 mV vs. SCE. The response of the electrode was recorded in a buffer solution (0.5 M TRIS-HCl buffer, pH = 8.8) supplemented with 2 mM hexacyanoferrate (III), for various ethanol concentrations. The relationship between ethanol concentration and current change is shown in Figure 17.

Example 17

Preparation of a "bulk" biosensor for the determination of ethanol, containing cholesteryl myristate as solid bindig maker, supplementing the tested solutions with a chemical mediator.

[0087] NAD (5 mg), diaphorase (4 mg), ADH (2 mg) and graphite powder (60 mg) were thoroughly mixed in a mortar; to the obtained mixture, cholesteryl myristate

(95 mg) was added and mixed. Chloroform (80 µl) was added and the mixture was vigorously stirred until the solvent was evaporated. The final product was introduced into a PVC tube, having an inner diameter of 2 mm, and pressed with a metal rod. The surface was then smoothed on a sheet of common paper and covered with a dialysis membrane, as described above. The obtained electrode was polarized at 300 mV vs. SCE. The response of the electrode was recorded in a buffer solution (0.5 M TRIS-HCl buffer, pH = 8.8), supplemented with 2 mM hexacyanoferrate (III), for various ethanol concentrations. The relationship between ethanol concentration and current change is shown in Figure 18.

Example 18

Preparation of a "bulk" biosensor for the determination of glucose, containing hexadecanone as solid binding maker.

[0088] 1 g of graphite powder was added to 62 mg of ferrocene, as described in Example 1. 450 mg of the obtained product were thoroughly mixed with a solution of glucose oxidase (50 mg of glucose oxidase in 0.5 ml of water) and water was evaporated under reduced pressure. 53 mg of the mixture was furtherly mixed with melted hexadecanone (70 mg), at the temperature of 50°C, and the obtained mixture was introduced into a PVC tip, cooled, smoothed and finally covered with a dialysis membrane, as described in example 12. The obtained electrode was polarized at 300 mV vs. SCE. The response of the electrode was recorded in a buffer solution (0.2 M sodium phosphate, pH = 8), at several glucose concentrations. The relationship between glucose concentration and current change is shown in Figure 19 (curve 1).

Example 19

Preparation of a "bulk" biosensor for the determination of glucose, containing eicosane as solid binding maker.

[0089] A biosensor for the determination of glucose was prepared as described in example 18, with the exception of using eicosane as solid binding maker instead of hexadecanone. The obtained electrode was polarized at 300 mV vs. SCE. The response of the electrode was recorded in a buffer solution (0.2 M sodium phosphate, pH = 8), at several glucose concentrations. The relationship between glucose concentration and current change is shown in Figure 19 (curve 2).

Claims

1. An electrochemical biosensor comprising:

a) at least an electro-conducting material, in the form of powder or grains;
 b) at least a chemical mediator;
 c) optionally, a substance capable of sorption of said chemical mediator;
 d) a solid binding maker selected from the group of chemical compounds consisting of: linear or branched, saturated or unsaturated hydrocarbons, containing from 12 to 60 carbon atoms, optionally substituted with at least a group selected from -OH, SH, -NH₂, -CO-, -CHO, SO₃H, -COOH, OR₁, -SR₁, NR₁R₂, and COOR₁, wherein R₁ and R₂ are independently hydrocarbon groups C₁-C₃₀, optionally containing one or more heteroatoms; esters of fatty acids with glycerol, and esters of fatty acids with cholesterol, wherein said solid binding maker which is in a solid state at room temperature, having a melting point comprised between 25°C and 200°C, and
 e) at least a biocatalyst, selected from the group consisting of enzymes, cells, cellular components, tissues, immunoproteins and DNA.

2. The electrochemical biosensor according to claim 1, characterized by the fact that said biocatalyst is incorporated into the bulk of the composite transducer constituted of the components (a)-(d).
3. The electrochemical biosensor according to claim 1, characterized by the fact that said biocatalyst is applied, in the form of a layer, onto the surface of the composite transducer constituted of the components (a)-(d).
4. The electrochemical biosensor according to anyone of claims 1-3, characterized by being covered by a suitable membrane.
5. The electrochemical biosensor according to claim 1, characterized by the fact that said electro-conducting material has a granulometry ranging from 0.05 to 200 µm.
6. The electrochemical biocatalyst according to claims 1 and 5, characterized by the fact that said electro-conducting material is a metal selected from the group consisting of gold, platinum, palladium, iridium and alloys thereof.
7. The electrochemical biosensor according to claims 1 and 5, characterized by the fact that said electro-conducting material is carbon or graphite.
8. The electrochemical biosensor according to claim 1, characterized by the fact that said chemical mediator is selected from the group consisting of cyto-

- chromes, quinones, aminophenols, electronacceptor aromatic compounds, electrondonor aromatic compounds, organic conducting salts, organic dyes, metallocenes, organometallic complexes of Os, Ru and V, and inorganic complexes of Fe. 5
9. The electrochemical biosensor according to claim 8, characterized by the fact that said chemical mediator is selected from the group consisting of TTF, NMP, TCNQ, TTF.TCNQ, NMP.TCNQ, ferrocene, 1,1'-dimethyl-ferrocene, hexacyanoferrate (II) and hexacyanoferrate (III). 10
10. The electrochemical biosensor according to claim 1, characterized by the fact that said substance capable of sorption of the chemical mediator is selected from the group consisting of silica gel, alumina, zeolites and Nafion®. 15
11. The electrochemical biosensor according to claim 1, characterized by the fact that said solid binding maker is selected from the group consisting of hexadecanol, hexadecanone, tetradecylamine, eicosane and tetracosane. 20
12. The electrochemical biosensor according to claim 1, characterized by the fact that said solid binding maker is an ester of glycerol with fatty acids, selected from monostearoyl glycerol and lecithin. 25
13. The electrochemical biosensor according to claim 1, characterized by the fact that said solid binding maker is an ester of cholesterol with a fatty acid, selected from the group consisting of cholesteryl myristate, cholesteryl stearate and cholesteryl oleate. 30
14. The electrochemical biosensor according to claim 1, characterized by the fact that said biocatalyst is an enzyme, selected from the group consisting of glucose oxidase, galactose oxidase, glycolate oxidase, alcohol oxidase, cholesterol oxidase, polyphenol oxidase, ascorbate oxidase, lipoxygenase, lipoxidase, peroxidase, catalase, xanthine oxidase, pyruvate oxidase, β-galactosidase, invertase, cholinesterase, citrate lyase, amylases and mixtures thereof. 35
15. The electrochemical biosensor according to claim 1, characterized by the fact that said biocatalyst is an enzyme requiring the presence of a co-factor, in association with said co-factor, selected from the group consisting of glucose dehydrogenase, alcohol dehydrogenase, fructose dehydrogenase; malate dehydrogenase, lactate dehydrogenase, mannitol dehydrogenase, glycerol dehydrogenase, isocitrate dehydrogenase, galactose dehydrogenase, glucose phosphate dehydrogenase, tartrate 40
- 55
- dehydrogenase and mixtures thereof.
16. The electrochemical biosensor according to claim 15, characterized by the fact that said co-factor is selected from the group consisting of NAD, NADH, NADP, NADPH, FAD, FMM and quinones.
17. The electrochemical biosensor according to claim 1, characterized by the fact that said biocatalyst is composed of cells, selected from the group consisting of *Gluconobacter oxidans*, *Escherichia coli*, *Aspergillus niger*, *Pseudomonas fluorescens*, *Trichosporon brassicae*, *Saccharomyces cerevisiae*, *Brevibacterium lactofermentum*, *Enterobacter agglomerans*, *Leuconostoc mesenteroides*, *Nocardia erythropolis* and mixtures thereof.
18. The electrochemical biosensor according to claim 4, characterized by the fact that said membrane is selected from the group consisting of dialysis membranes and membranes based on cellulose acetate, cellophane®, nitrocellulose, PVC, Teflon®, Nylon, polycarbonates and polyesters.
19. The electrochemical biosensor according to claim 1, characterized by comprising, with reference to 100 parts by weight of the mixture of the components (a)-(d):
- a) electro-conducting material: from 20 to 80% by weight;
 - b) chemical mediator: from 0.5 to 30% by weight;
 - c) substance capable of sorption of said chemical mediator: from 0 to 30% by weight;
 - d) solid binding maker: from 10 to 80% by weight.
20. The electrochemical biosensor according to claim 19, characterized by comprising, with reference to 100 parts by weight of the mixture of the components (a)-(d):
- a) electro-conducting material: from 30 to 60% by weight;
 - b) chemical mediator: from 1 to 10% by weight;
 - c) substance capable of sorption of said chemical mediator: from 1 to 20% by weight;
 - d) solid binding maker: from 30 to 60% by weight.
21. The electrochemical biosensor according to claims 1, 10, 19 and 20, characterized by the fact that said substance capable of binding the chemical mediator is contained in a quantity ranging from 0 to 30% by weight, when it is silica, alumina or zeolites, and from 0 to 5% by weight, when it is Nafion®, with reference to 100 parts by weight of the mixture of the

- components (a)-(d).
22. The electrochemical biosensor according to claim 2, characterized by the fact that said biocatalyst incorporated into the bulk of the composite transducer is contained in a quantity ranging from 1 to 30% by weight, with reference to the weight of said transducer. 5
23. A process for the preparation of a biosensor as described in anyone of claims 1-22, comprising the following steps: 10
- 1) mixing an electro-conducting material with a chemical mediator; 15
 - 2) optionally mixing the mixture obtained in step (1) with a substance capable of sorption of said chemical mediator;
 - 3) optionally mixing the mixture obtained in step (1) or (2) with a biocatalyst;
 - 4) suitably mixing the mixture obtained in step (1), (2) or (3) with a solid binding maker;
 - 5) introducing and optionally pressing the mixture obtained in step (4) in a suitable holder, thus obtaining a compact form;
 - 6) when step (3) is not worked out, applying a biocatalytic layer onto the surface of the transducer obtained in step (5);
 - 7) optionally covering the biosensor obtained in step (5) or (6) with a suitable membrane.
24. The process according to claim 23, characterized by the fact that, in step (4), said solid binding maker is mixed with the mixture obtained in step (1), (2) or (3) according to one of the following procedures: 35
- vigorous mechanical mixing of the solid components;
 - mixing in the presence of a suitable solvent which is then evaporated; 40
 - mixing with the solid binding maker in melted state.
25. A procedure for the determination of analytes concentration in a solution or a suspension, comprising: applying suitable electrode potential; contacting a biosensor, as described in anyone of claims 1-22, with said solution or suspension; and finally measuring current changes, which are proportional to the concentration of said analytes. 45
26. The procedure according to claim 25, characterized by the fact that the chemical mediator is contained in the bulk of said biosensor and/or in said sample solution or suspension. 50
27. The procedure according to claim 25, useful for the determination of analytes concentration in human
- and veterinary diagnostics, in industrial processes, in the quality control of food, in biotechnology, in the pharmaceutical industry and in environmental monitoring.
- Patentansprüche**
1. Elektrochemischer Biosensor, der umfaßt
 - a) mindestens ein elektrisch leitendes Material in Form eines Pulvers oder in Form von Körnchen (Granulat);
 - b) mindestens einen chemischen Vermittler (Mediator);
 - c) gegebenenfalls eine Substanz, die den genannten chemischen Vermittler sorbieren kann;
 - d) einen festen Bindungs-Bildner, ausgewählt aus der Gruppe von chemischen Verbindungen, die besteht aus linearen oder verzweigten, gesättigten oder ungesättigten Kohlenwasserstoffen, die 12 bis 60 Kohlenstoffatome enthalten, gegebenenfalls substituiert durch mindestens eine Gruppe, ausgewählt aus -OH, -SH, -NH₂, -CO-, -CHO, -SO₃H, -COOH, -OR₁, -SR₁, -NR₁R₂ und -COOR₁, worin R₁ und R₂ jeweils unabhängig voneinander C₁-C₃₀-Kohlenwasserstoffgruppen darstellen, die gegebenenfalls ein oder mehr Heteroatome enthalten; Estern von Fettsäuren mit Glycerin und Estern von Fettsäuren mit Cholesterin, wobei der genannte feste Bindungs-Bildner, der bei Raumtemperatur in einem festen Zustand vorliegt, einen Schmelzpunkt zwischen 25 und 200°C aufweist, und
 - e) mindestens einen Biokatalysator, ausgewählt aus der Gruppe, die besteht aus Enzymen, Zellen, Zellkomponenten, Geweben, Immunproteinen und DNA.
 2. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß der genannte Biokatalysator der Masse des Verbund-Wandlers einverleibt wird, der aus den Komponenten (a) bis (d) besteht.
 3. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß der genannte Biokatalysator in Form einer Schicht auf die Oberfläche des Verbundwandlers aufgebracht wird, der aus den Komponenten (a) bis (d) besteht.
 4. Elektrochemischer Biosensor nach einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß er von einer geeigneten Membran bedeckt ist.
 5. Elektrochemischer Biosensor nach Anspruch 1, da-

- durch gekennzeichnet, daß das genannte elektrisch leitende Material eine Korngröße in dem Bereich von 0,05 bis 200 µm aufweist.
6. Elektrochemischer Biosensor nach den Ansprüchen 1 und 5, dadurch gekennzeichnet, daß das elektrisch leitende Material ein Metall ist, das ausgewählt wird aus der Gruppe, die besteht aus Gold, Platin, Palladium, Iridium und Legierungen davon. 5
7. Elektrochemischer Biosensor nach den Ansprüchen 1 und 5, dadurch gekennzeichnet, daß das genannte elektrisch leitende Material Kohlenstoff oder Graphit ist. 10
8. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß der genannte chemische Vermittler ausgewählt wird aus der Gruppe, die besteht aus Cytochromen, Chinonen, Aminophenolen, aromatischen Elektronenakzeptor-Verbindungen, aromatischen Elektronendonator-Verbindungen, organischen Leitersalzen, organischen Farbstoffen, Metallocenen, metallorganischen Komplexen von Os, Ru und V und anorganischen Komplexen von Fe. 15
9. Elektrochemischer Biosensor nach Anspruch 8, dadurch gekennzeichnet, daß der genannte chemische Vermittler ausgewählt wird aus der Gruppe, die besteht aus TTF, NMP, TCNQ, TTF.TCNQ, NMP.TCNQ, Ferrocen, 1,1'-Dimethylferrocen, Hexacyanoferat(II) und Hexacyanoferat(III). 20
10. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß die genannte Substanz, die den chemischen Vermittler sorbieren kann, ausgewählt wird aus der Gruppe, die besteht aus Silicagel, Aluminiumoxid, Zeolithen und Nafion®. 25
11. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß der genannte feste Bindungs-Bildner ausgewählt wird aus der Gruppe, die besteht aus Hexadecanol, Hexadecanon, Tetradecylamin, Eicosan und Tetracosan. 30
12. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß der genannte feste Bindungs-Bildner ein Ester von Glycerin mit Fettsäuren ist, ausgewählt aus Monostearoylglycerin und Lecithin. 35
13. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß der genannte feste Bindungs-Bildner ein Ester von Cholesterin mit einer Fettsäure ist, ausgewählt aus der Gruppe, die besteht aus Cholestrylmyristat, Cholestrylstearat und Cholestryloleat. 40
14. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß der genannte Biokatalysator ein Enzym ist, ausgewählt aus der Gruppe, die besteht aus Glucoseoxidase, Galactoseoxidase, Glycolatoxidase, Alkoholoxidase, Cholesterin-oxidase, Polyphenoloxidase, Ascorbatoxidase, Lipooxygenase, Lipoxidase, Peroxidase, Catalase, Xanthinoxidase, Pyruvatoxidase, β-Galactosidase, Invertase, Cholinesterase, Citratlyase, Amylasen und Mischungen davon. 45
15. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß der genannte Biokatalysator ein Enzym, das die Anwesenheit eines Co-Faktors erfordert, in Assoziation mit dem genannten Co-Faktor ist, ausgewählt aus der Gruppe, die besteht aus Glucosedehydrogenase, Alkoholdehydrogenase, Fructosedehydrogenase, Malatdehydrogenase, Lactatdehydrogenase, Mannitdehydrogenase, Glycerindehydrogenase, Isocitratdehydrogenase, Galactosedehydrogenase, Glucosephosphatdehydrogenase, Tartratdehydrogenase und Mischungen davon. 50
16. Elektrochemischer Biosensor nach Anspruch 15, dadurch gekennzeichnet, daß der genannte Co-Faktor ausgewählt wird aus der Gruppe, die besteht aus NAD, NADH, NADP, NADPH, FAD, FMM und Chinonen. 55
17. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß der genannte Biokatalysator aus Zellen besteht, die ausgewählt werden aus der Gruppe, die besteht aus Gluconobacter oxidans, Escherichia coli, Aspergillus niger, Pseudomonas fluorescens, Trichosporon brassicae, Saccharomyces cerevisiae, Brevibacterium lactofermentum, Enterobacter agglomerans, Leuconostoc mesenteroides, Nocardia erythropolis und Mischungen davon.
18. Elektrochemischer Biosensor nach Anspruch 4, dadurch gekennzeichnet, daß die genannte Membran ausgewählt wird aus der Gruppe, die besteht aus Dialysemembranen und Membranen auf Basis von Celluloseacetat, Cellophan®, Nitrocellulose, PVC, Teflon®, Nylon, Polycarbonaten und Polyester. 60
19. Elektrochemischer Biosensor nach Anspruch 1, dadurch gekennzeichnet, daß er, bezogen auf 100 Gew.-Teile der Mischung der Komponenten (a) bis (d), umfaßt:
- a) 20 bis 80 Gew.-% des elektrisch leitenden Materials;
 - b) 0,5 bis 30 Gew.-% des chemischen Vermittlers (Mediators);
 - c) 0 bis 30 Gew.-% einer Substanz, die den che-

- mischen Vermittler sorbieren kann; und
d) 10 bis 80 Gew.-% des festen Bindungs-Bildners.
- 20. Elektrochemischer Biosensor nach Anspruch 19,** dadurch gekennzeichnet, daß er, bezogen auf 100 Gew.-Teile der Mischung der Komponenten (a) bis (d), umfaßt:
- a) 30 bis 60 Gew.-% des elektrisch leitenden Materials;
 - b) 1 bis 10 Gew.-% des chemischen Vermittlers (Mediators);
 - c) 1 bis 20 Gew.-% der Substanz, die den chemischen Vermittler sorbieren kann; und
 - d) 30 bis 60 Gew.-% des festen Bindungs-Bildners.
- 21. Elektrochemischer Biosensor nach den Ansprüchen 1, 10, 19 und 20,** dadurch gekennzeichnet, daß die genannte Substanz, die den chemischen Vermittler (Mediator) binden kann, in einer Menge in dem Bereich von 0 bis 30 Gew.-% darin enthalten ist, wenn es sich dabei um Siliciumdioxid, Aluminiumoxid oder Zeolithe handelt, und in einer Menge von 0 bis 5 Gew.-% darin enthalten ist, wenn es sich dabei um Nafion® handelt, jeweils bezogen auf 100 Gew.-Teile der Mischung der Komponenten (a) bis (d).
- 22. Elektrochemischer Biosensor nach Anspruch 2,** dadurch gekennzeichnet, daß der genannte Biokatalysator, welcher der Masse des Verbundwandlers einverleibt worden ist, in einer Menge in dem Bereich von 1 bis 30 Gew.-%, bezogen auf das Gewicht des Wandlers, darin enthalten ist.
- 23. Verfahren zur Herstellung eines Biosensors,** wie er in einem der Ansprüche 1 bis 22 beschrieben ist, das die folgenden Stufen umfaßt:
- 1) Mischen des elektrisch leitenden Materials mit einem chemischen Vermittler (Mediator);
 - 2) gegebenenfalls Mischen der in der Stufe (1) erhaltenen Mischung mit einer Substanz, die den chemischen Vermittler (Mediator) sorbieren kann;
 - 3) gegebenenfalls Mischen der in der Stufe (1) oder (2) erhaltenen Mischung mit einem Biokatalysator;
 - 4) geeignetes Mischen der in der Stufe (1), (2) oder (3) erhaltenen Mischung mit einem festen Bindungs-Bildner;
 - 5) Einführen und gegebenenfalls Pressen der in der Stufe (4) erhaltenen Mischung in einem geeigneten Halter unter Bildung einer kompakten (komprimierten) Form;
 - 6) Aufbringen einer biokatalytischen Schicht
- auf die Oberfläche des in der Stufe (5) erhaltenen Wandlers, wenn die Stufe (3) nicht durchgeführt worden ist; und
7) gegebenenfalls Bedecken des in der Stufe (5) oder (6) erhaltenen Biosensors mit einer geeigneten Membran.
- 24. Verfahren nach Anspruch 23,** dadurch gekennzeichnet, daß in der Stufe (4) der genannte feste Bindungs-Bildner mit der in der Stufe (1), (2) oder (3) erhaltenen Mischung unter Anwendung der folgenden Verfahrensmaßnahmen gemischt wird:
- starkes mechanisches Durchmischen der Feststoff-Komponenten;
 - Mischen in Gegenwart eines geeigneten Lösungsmittels, das dann verdampft wird; und
 - Mischen mit dem festen Bindungs-Bildner im geschmolzenen Zustand.
- 25. Verfahren zur Bestimmung der Analyten-Konzentration in einer Lösung oder Suspension,** das umfaßt das Anlegen eines geeigneten Elektrodenpotentials; das Inkontaktbringen eines Biosensors, wie er in einem der Ansprüche 1 bis 22 beschrieben ist, mit der genannten Lösung oder Suspension; und schließlich das Messen der Stromänderungen, die proportional zur Konzentration der genannten Analyten sind.
- 26. Verfahren nach Anspruch 25,** dadurch gekennzeichnet, daß der chemische Vermittler (Mediator) in der Masse des genannten Biosensors und/oder in der genannten Probelösung oder -suspension enthalten ist.
- 27. Verfahren nach Anspruch 25,** das anwendbar ist zur Bestimmung der Analyten-Konzentration in Human- und Veterinär-Diagnostika, in industriellen Verfahren, bei der Lebensmittelqualitätskontrolle, in der Biotechnologie, in der pharmazeutischen Industrie und bei der Umweltüberwachung.
- 45 Revendications**
1. Biocapteur électrochimique comprenant:
- a) au moins une matière électro-conductrice, sous forme de poudre ou de grains;
 - b) au moins un médiateur chimique;
 - c) éventuellement, une substance capable de sorption dudit médiateur chimique;
 - d) un producteur de liant solide choisi dans le groupe formé par les composés chimiques composés d'hydrocarbures saturés ou insaturés, linéaires ou ramifiés, contenant de 12 à 60 atomes de carbone, éventuellement substitués

- par au moins un groupe choisi parmi -OH, -SH, -NH₂, -CO-, -CHO, -SO₃H, -COOH, -OR₁, -SR₁, -NR₁R₂ et -COOR₁, dans lesquels R₁ et R₂ sont indépendamment les uns des autres des groupes hydrocarbonés en C₁ à C₃₀, contenant éventuellement un ou plusieurs hétéroatomes; les esters d'acides gras avec le glycérol, et les esters d'acides gras avec le cholestérol, dans lesquels ledit producteur de liant solide, qui est à l'état solide à la température ambiante, ayant un point de fusion compris entre 25EC et 200EC; et
- e) au moins un biocatalyseur, choisi dans le groupe formé par les enzymes, les cellules, les composants cellulaires, les tissus, les immuno-protéines et l'ADN.
2. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ledit biocapteur est incorporé dans la masse du transducteur composite constitué par les composants (a) à (d).
3. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ledit biocatalyseur est appliqué, sous forme de couche, sur la surface du transducteur composite constitué des composants (a) à (d).
4. Biocapteur électrochimique selon l'une quelconque des revendications 1 à 3, caractérisé par ce qu'il est recouvert par une membrane appropriée.
5. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ladite matière électro-conductrice possède une granulométrie comprise entre 0,05 et 200 µm.
6. Biocatalyseur électrochimique selon les revendications 1 à 5, caractérisé par le fait que ladite matière électro-conductrice est un métal choisi dans le groupe formé par l'or, le platine, le palladium, l'iridium et leurs alliages.
7. Biocapteur électrochimique selon les revendications 1 et 5, caractérisé par le fait que ladite matière électro-conductrice est le carbone ou le graphite.
8. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ledit médiateur chimique est choisi dans le groupe formé par les cytochromes, les quinones, les aminophénols, les composés aromatiques accepteurs d'électrons, les composés aromatiques donneurs d'électrons, les sels organiques conducteurs, les colorants organiques, les métallocènes, les complexes organométalliques de Os, Ru et V, et les complexes inorganiques de Fe.
9. Biocapteur électrochimique selon la revendication 8, caractérisé par le fait que ledit médiateur chimique est choisi dans le groupe formé par TTF, NMP, TCNQ, TTF.TCNQ, NMP.TCNQ, le ferrocène, le 1,1'-diméthyl-ferrocène, l'hexacyanoferrate (II) et l'hexacyanoferrate (III).
10. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ladite substance capable de sorption du médiateur chimique est choisie dans le groupe formé par le gel de silice, l'alumine, les zéolites et Nafion®.
11. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ledit producteur de liant solide est choisi dans le groupe formé par l'hexadécanol, l'hexadécane, la tétradécyamine, l'éicosane et la tétracosané.
12. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ledit producteur de liant solide est un ester de glycérol avec des acides gras, choisi parmi le monostéaroyle glycérol et la lécithine.
13. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ledit producteur de liant solide est un ester de cholestérol avec un acide gras, choisi dans le groupe formé par le myristate de cholestéryle, le stéarate de cholestéryle et l'oléate de cholestéryle.
14. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ledit biocatalyseur est une enzyme, choisie dans le groupe formé par la glucose oxydase, la galactose oxydase, la glycolate oxydase, l'alcool oxydase, la cholestérol oxydase, la polyphénol oxydase, l'ascorbate oxydase, la lipoygénase, la lipoydase, la peroxydase, la catalase, la xanthine oxydase, la pyruvate oxydase, la β-galactosidase, l'invertase, la cholinestérase, la citrate lyase, les amylases et leurs mélanges.
15. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ledit biocatalyseur est une enzyme nécessitant la présence d'un co-facteur, en association avec ledit co-facteur, choisi dans le groupe formé par la glucose déhydrogénase, l'alcool déhydrogénase, la fructose déhydrogénase, la malate déhydrogénase, la lactate déhydrogénase, la mannitol déhydrogénase, la glycérol déhydrogénase, l'isocitrate déhydrogénase, la galactose déhydrogénase, la glucose phosphate déhydrogénase, la tartrate déhydrogénase et leurs mélanges.
16. Biocapteur électrochimique selon la revendication 15, caractérisé par le fait que ledit co-facteur est

- choisi dans le groupe formé par NAD, NADH, NADP, NADPH, FAD, FMM et les quinones.
17. Biocapteur électrochimique selon la revendication 1, caractérisé par le fait que ledit biocatalyseur est composé de cellules, choisies dans le groupe formé par *Gluconobacter oxidans*, *Escherichia coli*, *Aspergillus niger*, *Pseudomonas fluorescens*, *Trichosporon brassicae*, *Saccharomyces cerevisiae*, *Brevibacterium lactofermentum*, *Enterobacter agglomerans*, *Leuconostoc mesenteroides*, *Nocardia erythropolis* et leurs mélanges. 5
18. Biocapteur électrochimique selon la revendication 4, caractérisé par le fait que ladite membrane est choisie dans le groupe formé par les membranes de dialyse et les membranes à base d'acétate de cellulose, de cellophane®, de nitrocellulose, de PVC, de Teflon®, de Nylon, de polycarbonates et de polyesters. 10 15
19. Biocapteur électrochimique selon la revendication 1, caractérisé en ce qu'il comprend, en référence à 100 parties en poids du mélange des composants (a) à (d): 20 25
- a) une matière électro-conductrice: de 20 à 80% en poids;
 - b) un médiateur chimique: de 0,5 à 30% en poids;
 - c) une substance capable de sorption dudit médiateur chimique: de 0 à 30% en poids;
 - d) un producteur de liant solide: de 10 à 80% en poids.
20. Biocapteur électrochimique selon la revendication 19 , caractérisé en ce qu'il comprend, en référence à 100 parties en poids du mélange des composants (a) à (d): 30 35
- a) une matière électro-conductrice: de 30 à 60% en poids;
 - b) un médiateur chimique: de 1 à 10% en poids;
 - c) une substance capable de sorption dudit médiateur chimique: de 1 à 20% en poids;
 - d) un producteur de liant solide: de 30 à 60% en poids.
21. Biocapteur électrochimique selon les revendications 1, 10, 19 et 20, caractérisé par le fait que ladite substance capable de lier le médiateur chimique est contenue en une quantité comprise entre 0 et 30% en poids, quand c'est une silice, une alumine ou des zéolites, et de 0 à 5% en poids, quand c'est Nafion®, en référence à 100 parties en poids du mélange des composants (a) à (d). 40 45 50 55
22. Biocapteur électrochimique selon la revendication 2, caractérisé par le fait que ledit biocatalyseur incorporé dans la masse du transducteur composite est contenu à raison de 1 à 30% en poids, en référence au poids dudit transducteur.
23. Procédé pour la préparation d'un biocapteur comme décrit selon l'une quelconque des revendications 1 à 22, comprenant les étapes suivantes consistant: 10
- 1) à mélanger une matière électro-conductrice avec un médiateur chimique;
 - 2) à mélanger éventuellement le mélange obtenu dans l'étape (1) avec une substance capable de sorption dudit médiateur chimique;
 - 3) à mélanger éventuellement le mélange obtenu dans l'étape (1) ou (2) avec un biocatalyseur;
 - 4) à mélanger de façon appropriée le mélange obtenu dans l'étape (1), (2) ou (3) avec un producteur de liant solide;
 - 5) à introduire et éventuellement à presser le mélange obtenu dans l'étape (4) dans un support approprié, obtenant ainsi une forme compacte;
 - 6) quand l'étape (3) n'est pas réussie, à appliquer une couche biocatalytique sur la surface du transducteur obtenu dans l'étape (5);
 - 7) à recouvrir éventuellement le biocapteur obtenu dans l'étape (5) ou (6) avec une membrane appropriée.
24. Procédé selon la revendication 23, caractérisé par le fait que, dans l'étape (4), ledit producteur de liant solide est mélangé avec le mélange obtenu dans l'étape (1), (2) ou (3) selon l'un quelconque des procédés suivants: 30
- mélange mécanique vigoureux des composants solides;
 - mélange en présence d'un solvant approprié qui est ensuite évaporé;
 - mélange avec le producteur de liant solide à l'état fondu.
25. Procédé pour la détermination de concentrations d'analytes dans une solution ou une suspension, consistant: à appliquer un potentiel d'électrode approprié; à mettre en contact un biocapteur, comme décrit selon l'une quelconque des revendications 1 à 22, avec ladite solution ou suspension; et finalement à mesurer des variations de courant, qui sont proportionnelles à la concentration desdits analytes. 40
26. Procédé selon la revendication 25, caractérisé par le fait que le médiateur chimique est contenu dans la masse dudit biocapteur et/ou dans ladite solution

ou suspension échantillon.

27. Procédé selon la revendication 25, utile pour la détermination de concentrations d'analytes dans les diagnostics humains et vétérinaires, dans les procédés industriels, dans le contrôle qualité des aliments, en biotechnologie, dans l'industrie pharmaceutique et dans la surveillance de l'environnement.

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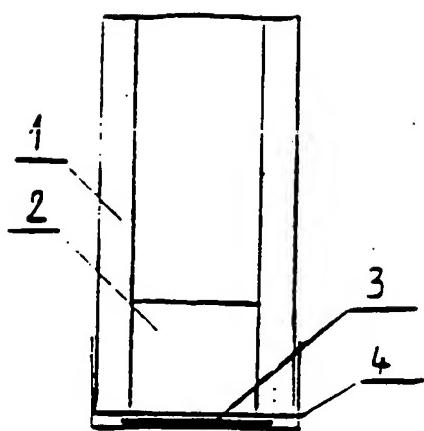


FIG. 1A

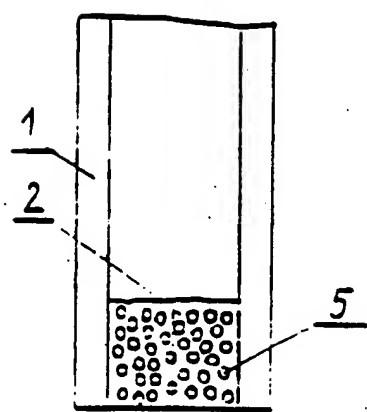


FIG. 1B

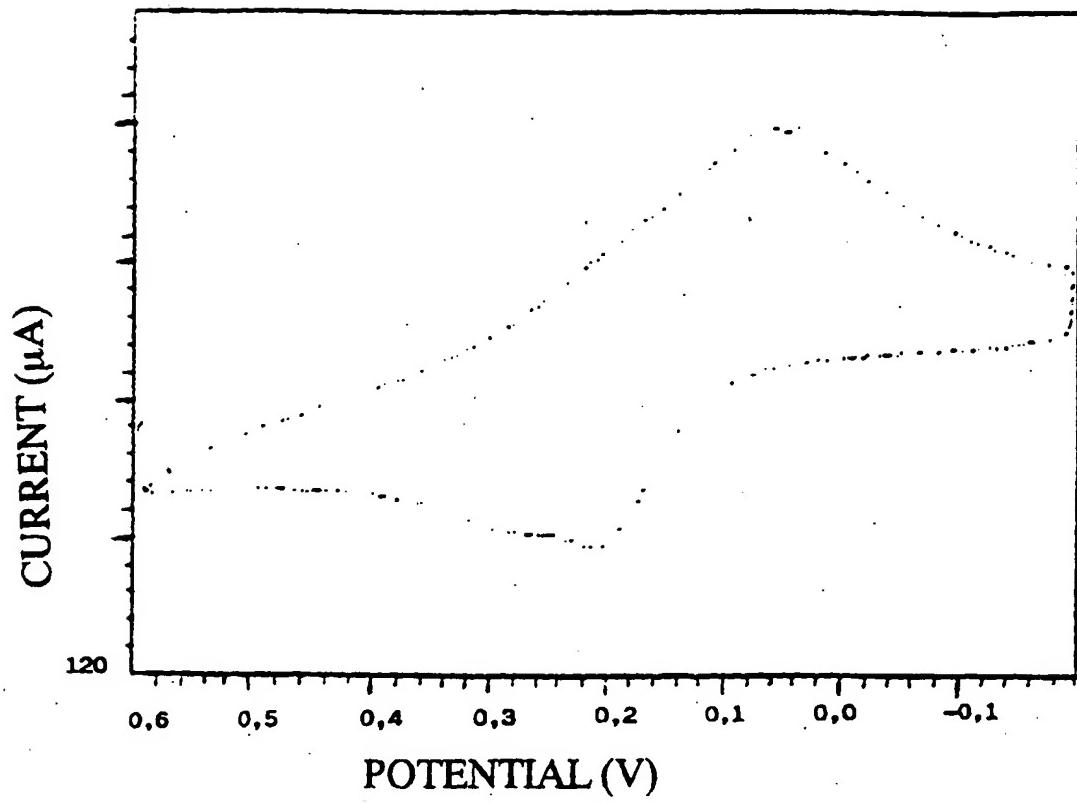


FIG. 2

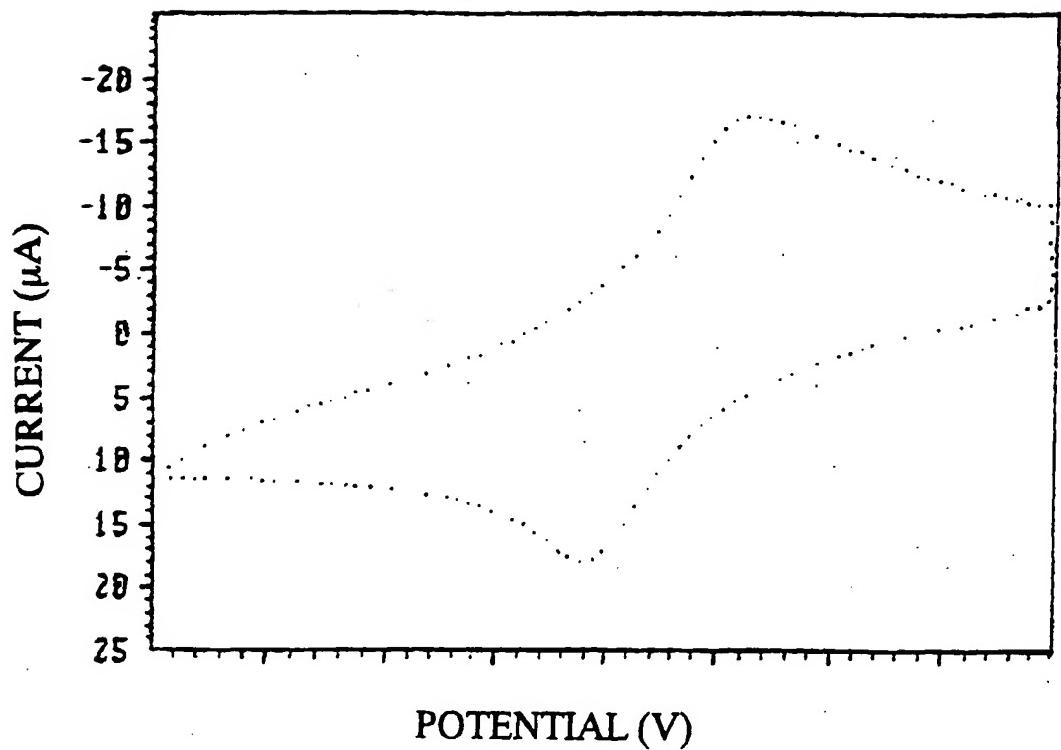


FIG. 3

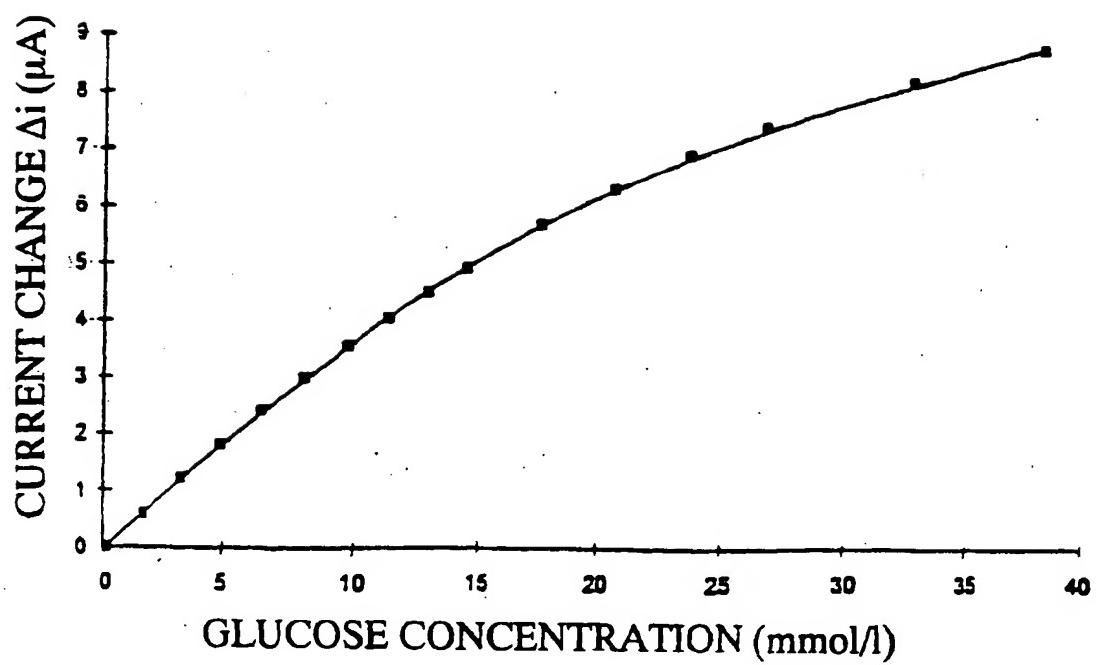


FIG. 4

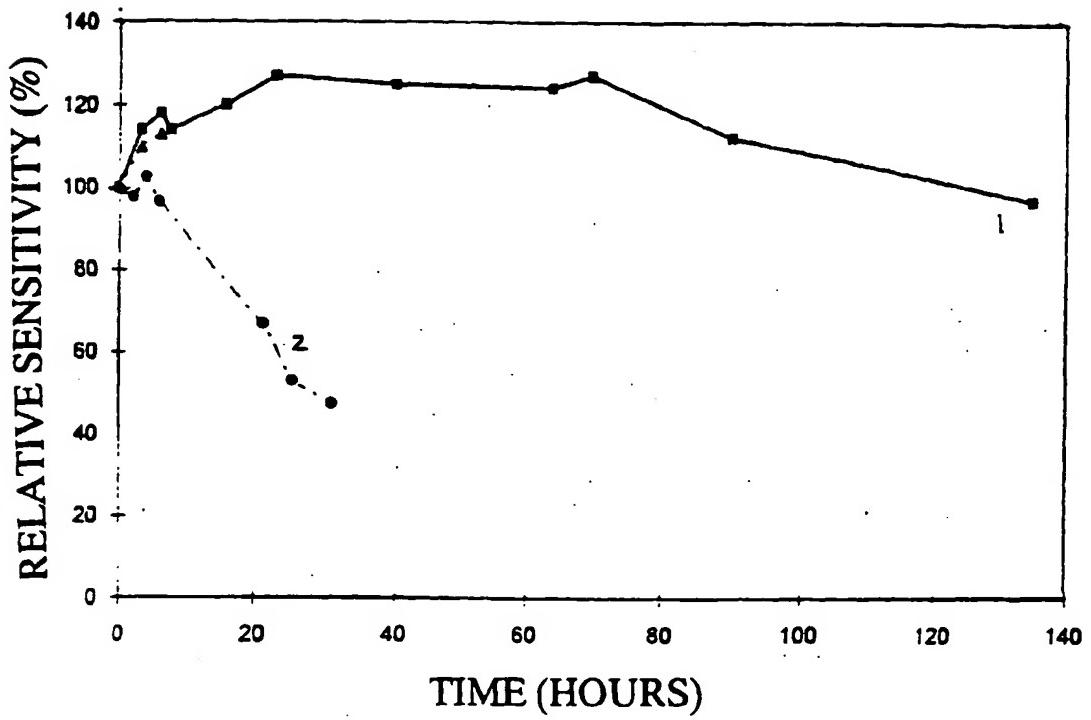


FIG. 5

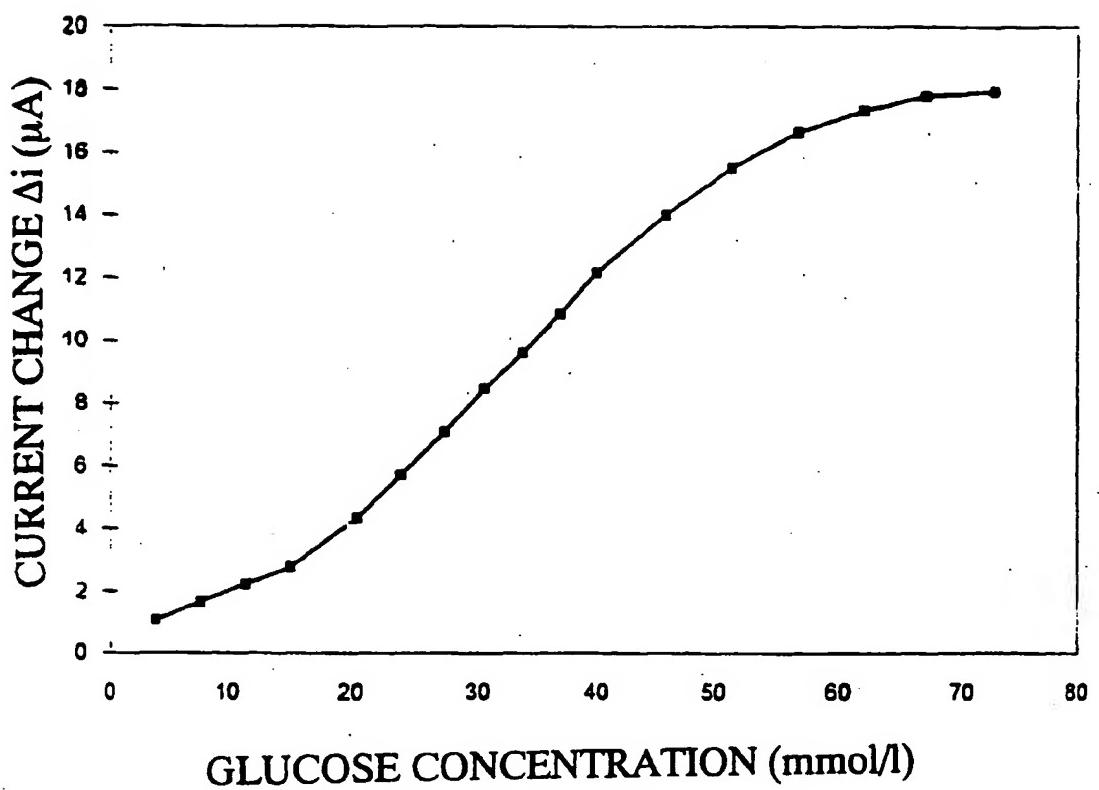


FIG. 6

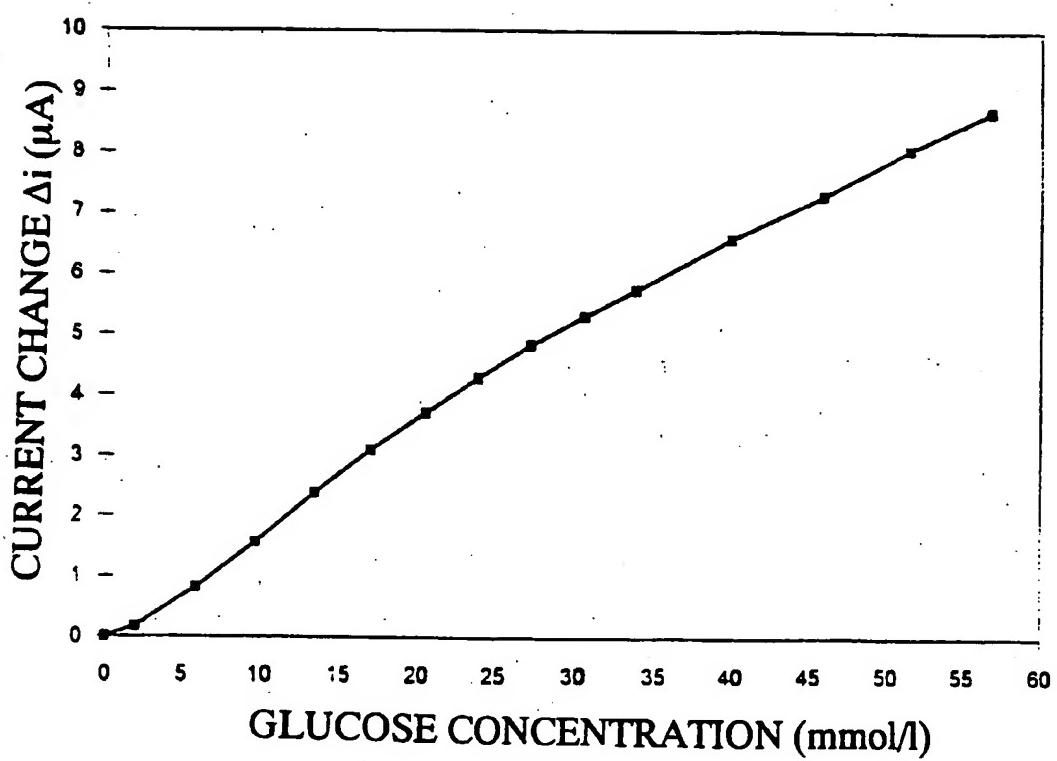


FIG. 7

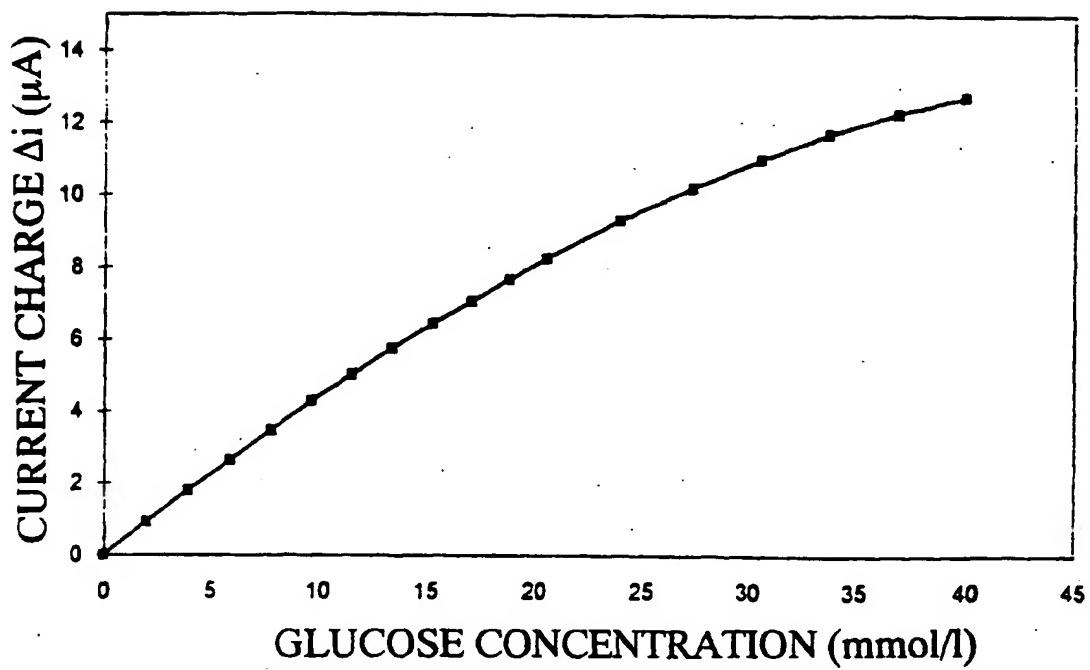


FIG. 8

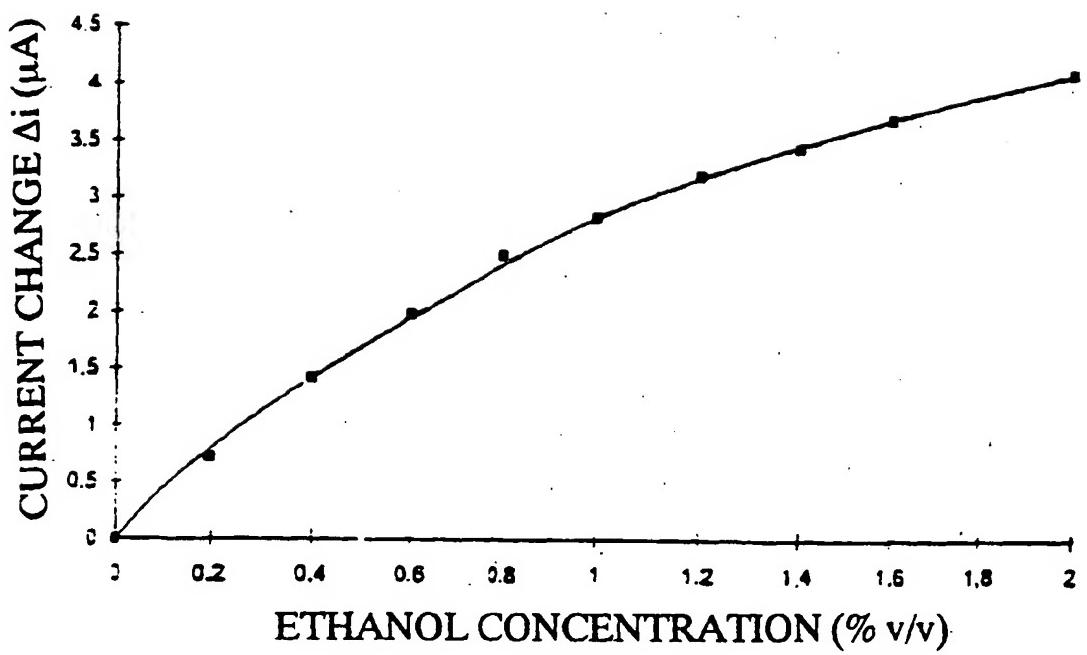


FIG. 9

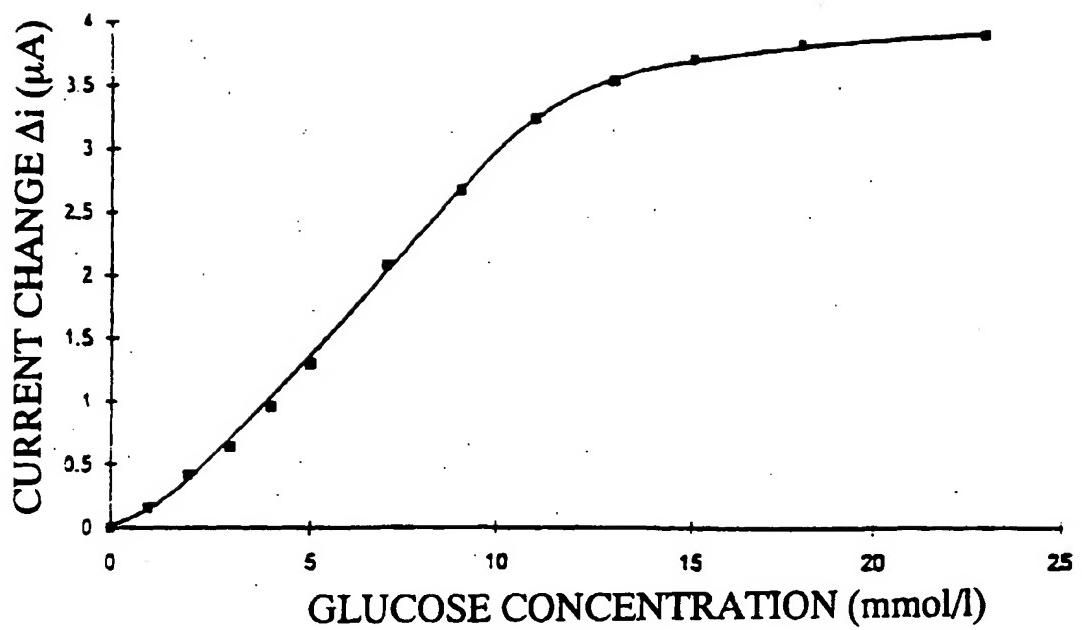


FIG. 10

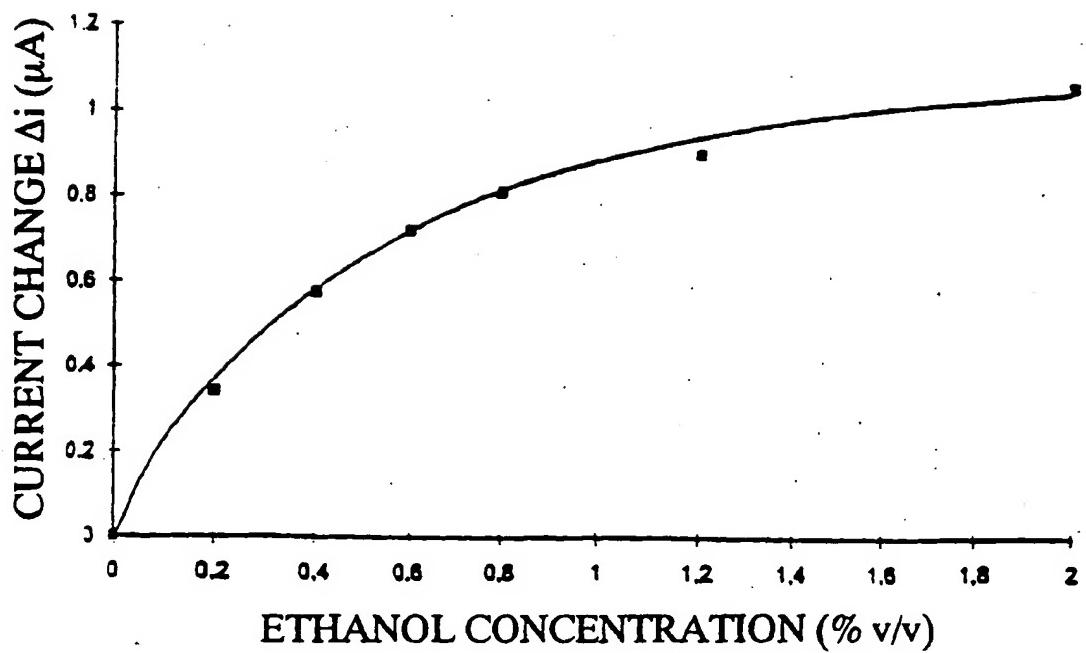


FIG. 11

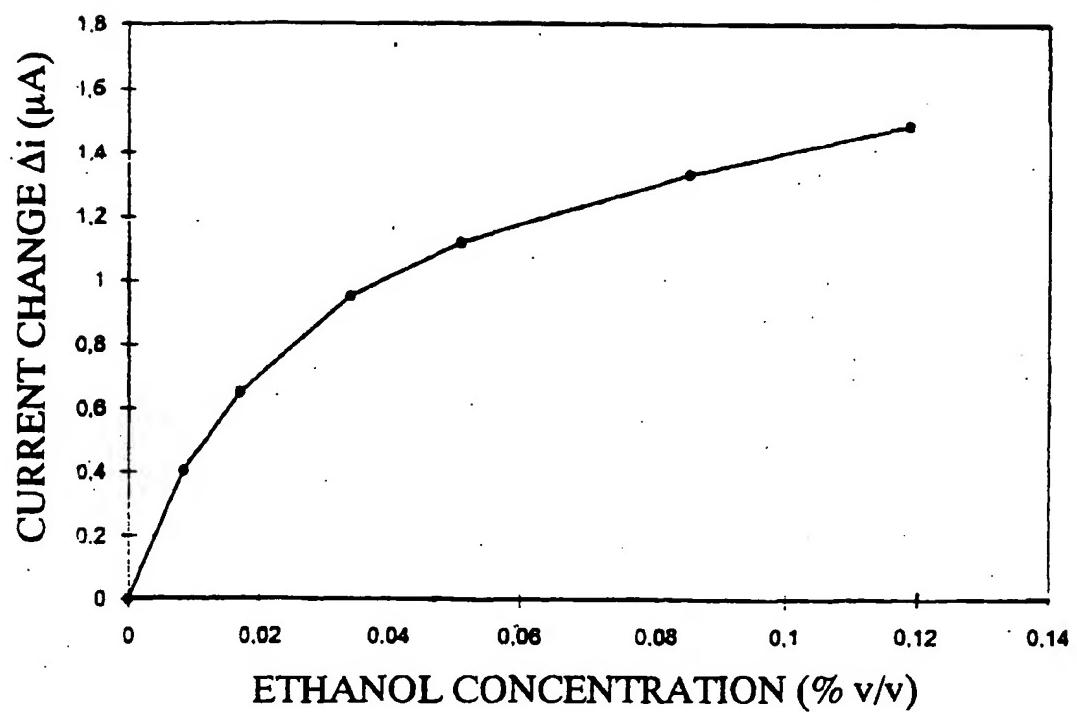


FIG. 12

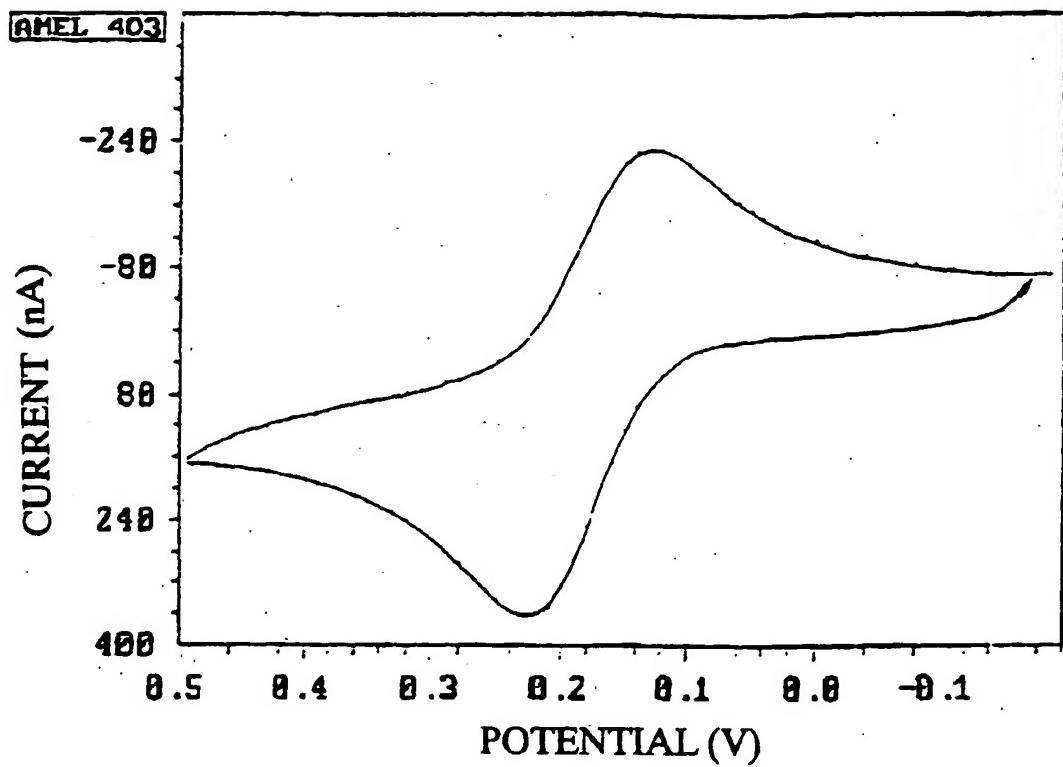


FIG. 13

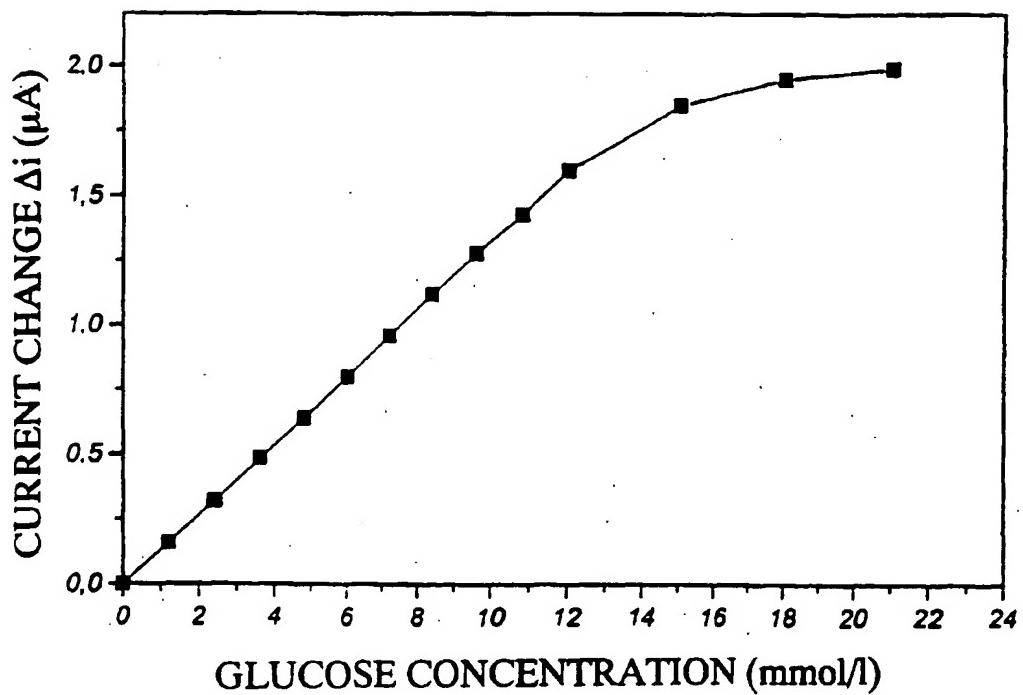


FIG. 14

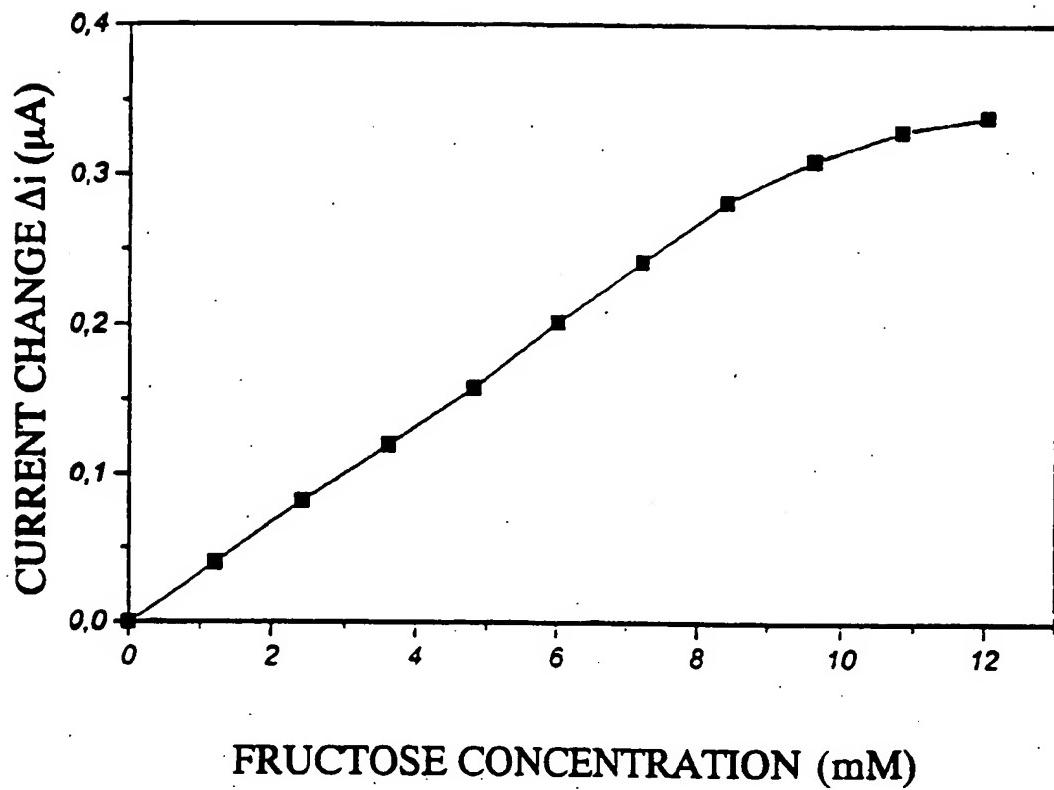


FIG. 15

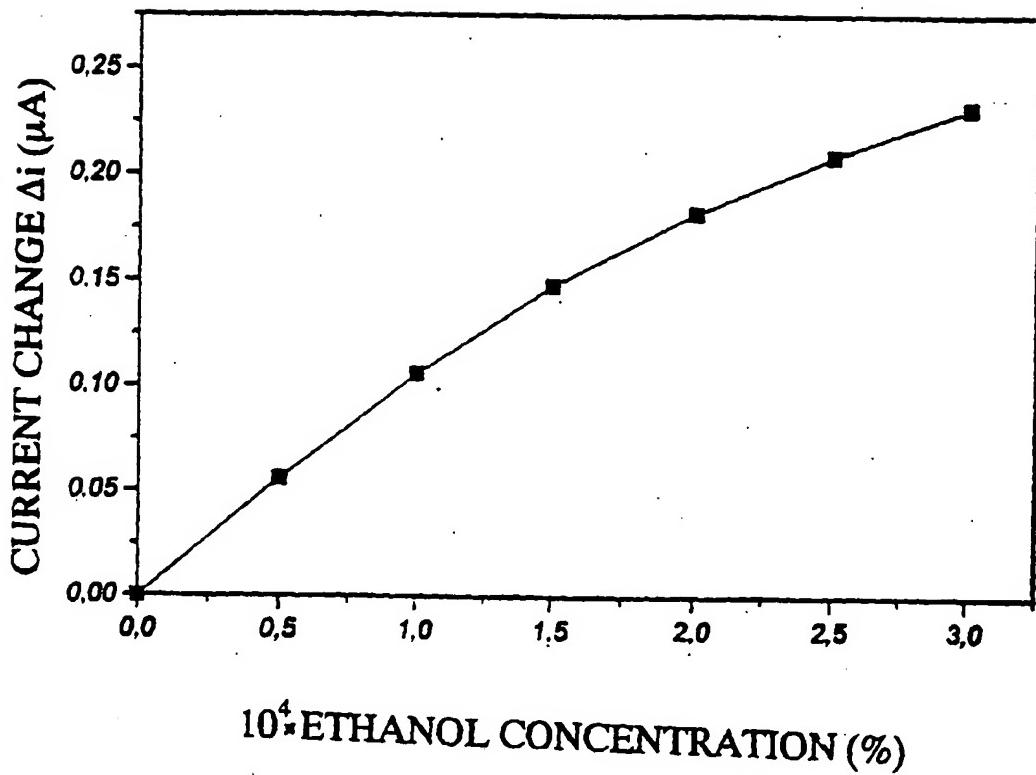


FIG. 16

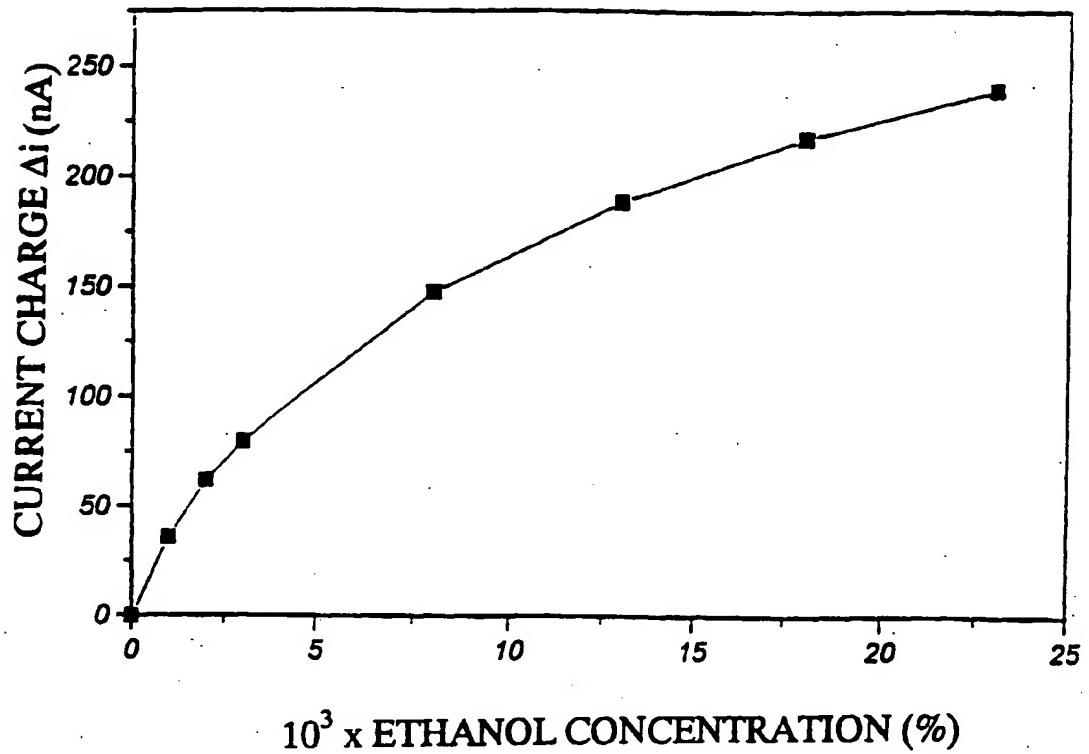


FIG. 17

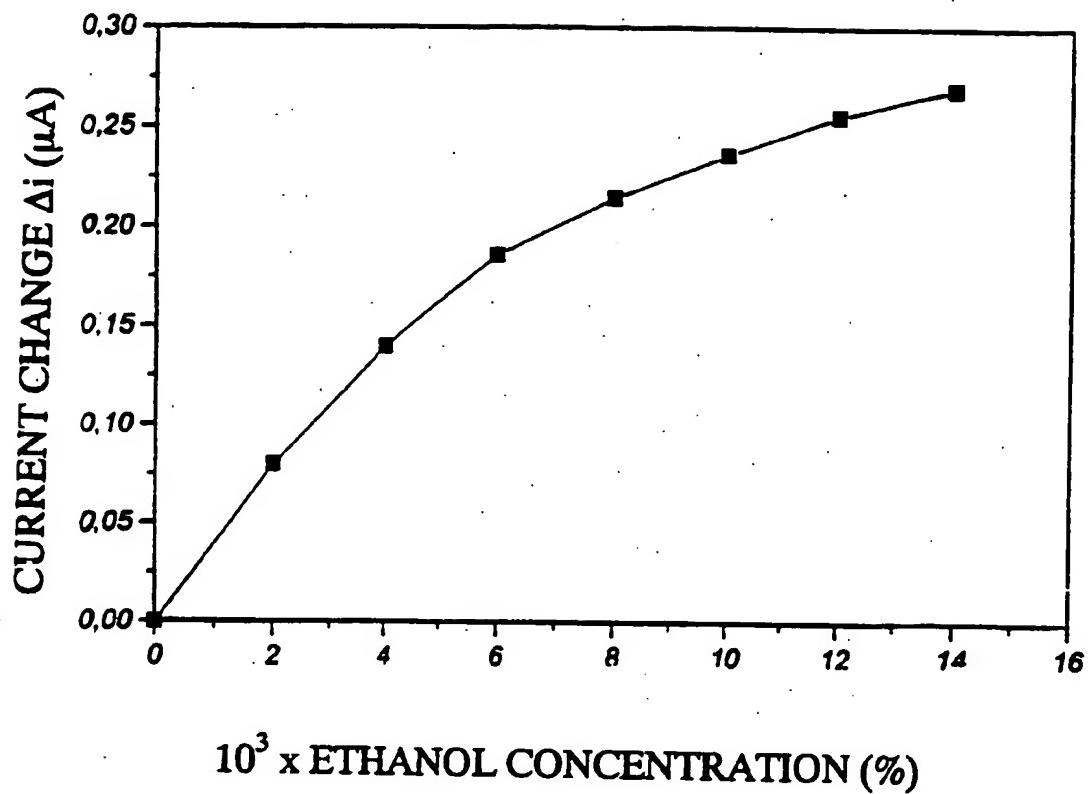


FIG. 18

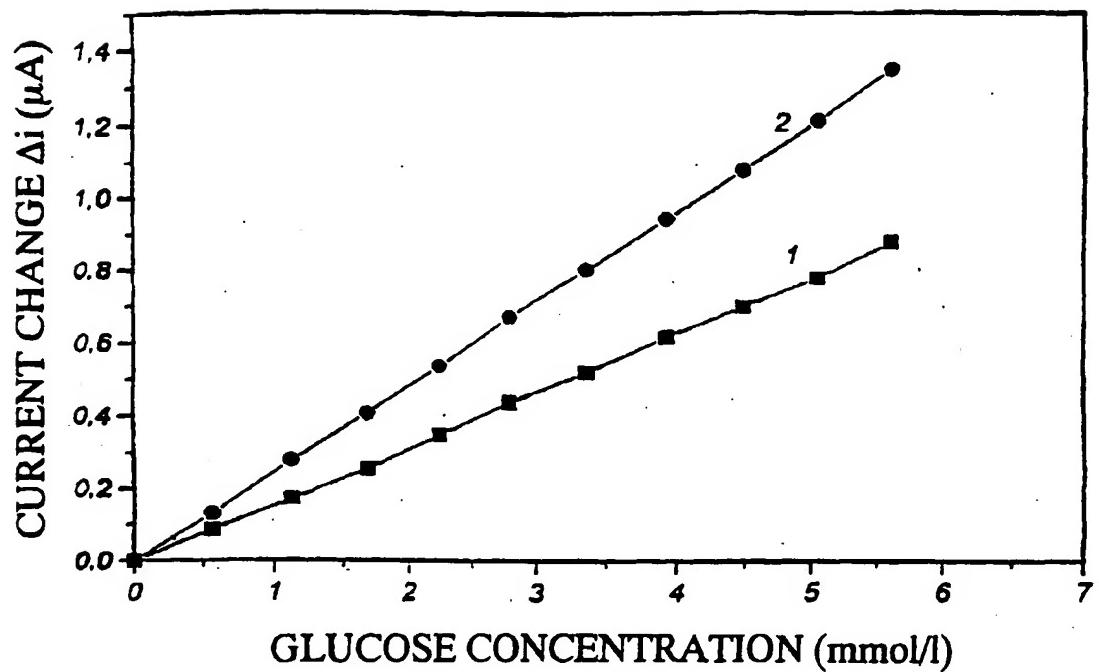


FIG. 19